Pharmacokinetic herb-drug interactions involving African traditional medicines - fingerprint analysis and in vitro metabolism studies

by

Saneesh Kumar

Dissertation submitted to the Faculty of Medicine and Health Sciences, Stellenbsoch University, in fulfilment of the requirements for the degree of Doctor of Philosophy (Pharmacology).

Supervisor: Bernd Rosenkranz
Professor, Division of Clinical Pharmacology, Faculty of Medicine and Health Sciences

Co-Supervisor: Patrick J. Bouic
Professor, Division of Medical Microbiology, Faculty of Medicine and Health Sciences

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2018

Abstract

Introduction

Traditional, complementary and alternative medicines have been used to treat various health conditions. The use of such medicines among HIV/AIDS and TB patients in sub-Saharan Africa has increased considerably, and within this context, questions have been raised about the medical use of herbs or extracts as treatment alternatives without adequate clinical testing and without monitoring of adverse effects once on the market. Furthermore, potential herb-drug interactions (HDI) have been predicted based on the pharmacological and pharmacokinetic properties of prescription medications and the phytoconstituents within the herbs.

This study investigated the potential of six popular African herbs consumed by HIV/AIDS and TB patients, *viz*., *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and *Ocimum basilicum*, to inhibit the cytochrome P450 enzyme CYP2B6 and the esterase-mediated metabolism pathway of rifampicin and their ability to induce CYP3A4 and CYP2B6. The study was undertaken in four phases: (1) qualitative assessment of various classes of phytocompounds present in each herbal extract by biochemical phytoprofiling, (2) study of the potential inhibitory effects of the extracts on cytochrome CYP2B6 and on the metabolism pathway of rifampicin to 25-O-desacetyl rifampicin by *in vitro* assays using human liver microsomes (HLM), (3) analysis of the potential inducing effects of the herbal extracts on mRNA expression of CYP2B6 and CYP3A4 in HepG2 cell lines, using reverse transcription polymerase chain reaction (RT-PCR) and agarose gel electrophoresis (AGE), and (4) fingerprint analysis, identification and relative quantification of the major phytoconstituents present in each extract and prediction of compounds which may cause HDI by liquid chromatography-mass spectrometry coupled with photo diode array detection (LC-MS/PDA).

Methods

Dried roots of *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and dried leaves and inflorescence of *Ocimum basilicum* were obtained from Pharma Germania, South Africa. Aqueous, methanol, ethanol and ethyl acetate extracts were prepared and analysed using biochemical tests to identify the

presence of various classes of phytocompounds. HLM assays were conducted to evaluate the inhibitory potential of each extract on the CYP2B6-mediated metabolism of efavirenz to 8-Hydroxy efavirenz, and the biotransformation of rifampicin to 25-O-desacetyl rifampicin. The protocol included the incubation of the herbal extract, HLM, co-factors and substrates in phosphate buffer for 30 min at 37 ^{o}C, termination of reaction and HPLC analysis of the supernatant from the centrifuged assay sample (at 245 nm for efavirenz and its metabolite, and 254 nm for rifampicin and its metabolite). The half-maximal inhibitory concentrations (IC_{50}) for the active extracts were calculated based on the percentage of remaining activity relative to the control. Time-dependent inhibition (TDI) IC_{50} fold-shift was evaluated using 30 min pre-incubation with NADPH, followed by incubation with substrate in buffer for another 30 min, using six concentrations (1-200 μg/mL) of the herbal extract. CYP2B6 and CYP3A4 mRNA expression assays were conducted for measuring the induction potential of the extracts, where the 50% cytotoxic concentration (CTC_{50}) of all herbal extracts was determined by screening them (1000.00-31.25 μg/mL) against HepG2 cells. The HepG2 cells were incubated for 24 h with the CTC_{50} concentration, for each herb. This was followed by extraction and purification of total mRNA and its expression through RT-PCR, followed by AGE. Relative sample expression levels were calculated and represented as fold-response levels of induction relative to a cell control (using rifampicin and dexamethasone as positive controls).
LC-MS/PDA was used to identify and relatively quantify the potential phytochemical constituents in each extract.

Results

O.basilicum, G.glabra I.helenium and *A.membranaceus* contained most of the relevant groups of phytocompounds such as flavonoids, phenols, alkaloids, glycosides and terpenoids based on the biochemical qualitative analysis. The aqueous and methanolic extracts of *O.basilicum* showed reversible and time-dependent inhibition of CYP2B6 (TDI IC_{50}s 33.35 μg/mL, 4.93 μg/mL, IC_{50} shift-fold >1.5 for both extracts), while the methanolic and ethanolic extracts inhibited the formation of 25-O-desacetyl rifampicin (IC_{50}s 31 μg/mL, 8.94 μg/mL). The methanolic extract of *O.basilicum* showed the highest TDI with a 7.4-fold increase in the IC_{50}. All extracts of *I.helenium* inhibited CYP2B6 (IC_{50}s 63 μg/mL, 89.43 μg/mL) and rifampicin metabolism (IC_{50}s 42.79 μg/mL, 18.58 μg/mL, 62.10 μg/mL); the aqueous extract showed the highest TDI with a 3-fold increase

in the IC_{50}. Only the methanolic and ethyl acetate extracts of *W.somnifera* inhibited CYP2B6 (IC_{50} 79.16 μg/mL, 57.96 μg/mL). TDI was mainly observed between the herbal extracts and CYP2B6.

The ethanolic and methanolic extracts of *A.officinalis* induced CYP3A4, with 48%-fold-response shift compared to the cell control (no inducer). The ethanolic extract of *O.basilicum* and *G.glabra* caused moderate induction of CYP3A4 and CYP2B6. The aqueous extract of *A.membranaceus* showed moderate and equal induction of CYP3A4 and CYP2B6 (36% fold-response increase).

All extracts exhibited less than 2-fold induction (200%) response, in contrast to the positive controls, rifampicin and dexamethasone.

Major phytocompounds detected in the LC-MS/PDA analysis of the extracts included flavonoids, phenols, glycosides, saponins and terpenoids. Relative amounts of the identified compounds were determined by comparison to standard calibrators, quercetin and gallic acid (expressed in mg/L equivalent units). Phenols such as rosmarinic acid (approximately 2298 mg/L in the aqueous extract) and caftaric acid were found in *O.basilicum* extracts along with the flavonoids salvigenin, rutin and isoquercetin and other compounds such as linalool, hydroxyjasmonic acid and eucommiol. The aqueous extract of *I.helenium* contained mostly polyphenols such as chlorogenic and caffeoylquinic acids, whereas other solvent extracts contained the sesquiterpenoid tanacetol A, helenin (isoalantolactone) and macrophyllilactone B. The methanolic and ethyl acetate extracts of *W.somnifera* comprised of withaperuvin, isopelletierine, salvigenin, withanolides and withaferin A (approximately 1117 mg/L in the ethyl acetate extract), and the extracts of *A.membranaceus* contained mainly calycosin, formononetin, astragalosides I and IV.

The extracts of *A.officinalis* mainly comprised of quinic acid and altheahexacosanyl lactone derivatives (approximately 3874 mg/L in the methanol extract), d-galacturonic acid monohydrate, phloretin along with the fatty acid trihydroxy-octadecenoic acid, and the *G.glabra* extract mainly consisted of glabridin, liquiritin apioside, liquiritigenin and licochalcones.

Conclusion

A.membranaceus, I.helenium, O.basilicum and *W.somnifera* have been shown to contain astragalosides, alantolactones, phenolic acids and withanolides that may inhibit drug metabolising enzymes such as CYP2B6, CYP3A4 and the enzymes responsible for

metabolism of rifampicin (including B-esterases). *A.officinalis* and *G.glabra* can cause moderate induction of CYP3A4 due to the higher concentration of lactones and chalcones present in their extracts.

These *in vitro* findings may be relevant for clinical use of these herbs together with conventional medicines metabolised by these enzymes, if sufficient hepatic concentrations are attained in humans.

The results of this study will help to guide planning and designing of clinical trials to confirm the potential relevance of HDI in patients.

Abstrak

Inleiding

Tradisionele, aanvullende en alternatiewe medisyne word gebruik om verskeie gesondheidstoestande te behandel. Die gebruik van sodanige medisyne vir MIV/vigs- en TB-pasiënte in Afrika suid van die Sahara het aanmerklik toegeneem, en in hierdie konteks word vrae gevra oor die medisinale gebruik van kruie of ekstrakte as behandelingalternatiewe sonder voldoende kliniese toetsing en sonder monitering van negatiewe gevolge nadat dit op die mark verskyn het. Moontlike kruiemedikasie-interaksies (KMIs) word voorts voorspel op grond van die farmakologiese en farmakokinetiese eienskappe van voorskrifmedikasie en die fitobestanddele in die kruie.

Hierdie studie was ’n ondersoek na die potensiaal van ses gewilde Afrika-kruie wat deur MIV/vigs- en TB-pasiënte gebruik word, naamlik *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* en *Ocimum basilicum*, om die sitochroom P450-ensiem CYP2B6 en die esterase-bemiddelde metabolismebaan van rifampisien en hul vermoë om CYP3A4 en CYP2B6 te induseer, te inhibeer. Die studie is in vier fases onderneem: (1) kwalitatiewe assessering van verskillende klasse van fitoverbindings teenwoordig in elke kruie-ekstrak deur biochemiese fitoprofilering, (2) bestudering van die potensiële inhiberende gevolge van die ekstrakte op sitochroom CYP2B6 en op die metabolismebaan van rifampisien tot 25-O-desasetiel-rifampisien deur *in vitro*-ontledings met gebruik van menslewermikrosome (MLM), (3) ontleding van die potensiële induserende uitwerkings van die kruie-ekstrakte op mRNA-uitdrukking van CYP2B6 en CYP3A4 in HepG2-sellyne met gebruik van omgekeerde transkripsie polimerasiekettingreaksie (RT-PCR) en agarose-jel-elektroforese (AGE), en (4) vingerafdrukontleding, -identifisering en relatiewe versyfering van die vernaamste fitobestanddele teenwoordig in elke ekstrak en voorspelling van verbindings wat KMI’s kan veroorsaak deur vloeibare chromatografie-massa-spektrometrie gepaard met fotodiode-rangskikkingwaarneming (LC-MS/PDA).

Metodes

Droë wortels van *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium* en *Althaea officinalis* en droë blare en infloressensie van *Ocimum basilicum* is van Pharma Germania, Suid-Afrika, verkry. Water-, metanol-, etanol- en

etielasetaat-ekstrakte is voorberei en ontleed met behulp van biochemiese toetse om die teenwoordigheid van verskillende klasse fitoverbindings te identifiseer. MLM-ontledings is uitgevoer om die inhiberende potensiaal van elke ekstrak op die CYP2B6-bemidelde metabolisme van efavirenz tot 8-hidroksie-efavirenz en die biotransformasie van rifampisien tot 25-O-desasetiel-rifampisien te evalueer. Die protokol het die inkubasie van die kruie-ekstrak, MLM, kofaktore en substrate in fosfaatbuffer vir 30 minute teen 37 ^{o}C, beëindiging van reaksie en hoëdruk- vloeibare chromatografie-ontleding van die bodrywende stof van die gesentrifugeerde toetsmonster (teen 245 nm vir efavirenz en sy metaboliet, en 254 nm vir rifampisien en sy metaboliet) ingesluit. Die half-maksimale inhiberende konsentrasies (IC_{50}) vir die aktiewe ekstrakte is bereken op grond van die persentasie oorblywende aktiwiteit in verhouding tot die kontrole. Tydafhanklike inhibisie (TAI) IC_{50}-verskuiwingsvou is geëvalueer deur 30 minute pre-inkubasie met NADPH, gevolg deur inkubasie met substraat in buffer vir ’n verdere 30 minute met die gebruik van ses konsentrasies (1-200 μg/mL) van die kruie-ekstrak. CYP2B6- en CYP3A4 mRNA-uitdrukkingontledings is uitgevoer om die induksiepotensiaal van die ekstrakte te meet, waar die 50% sitotoksiese konsentrasie (CTC_{50}) van alle kruie-ekstrakte bepaal is deur hulle teen HepG2-selle te toets (1000.00-31.25 μg/mL). Die HepG2-selle is vir 24 uur met die CTC_{50}-konsentrasie vir elke krui geïnkubeer. Dit is gevolg deur ekstraksie en suiwering van totale mRNA en sy uitdrukking deur RT-PCR, gevolg deur AGE. Relatiewe monsteruitdrukkingvlakke is bereken en as vouresponsvlakke van induksie in verhouding tot ’n selkontrole voorgestel (met gebruik van rifampisien en deksametasoon as positiewe kontroles). LC-MS/PDA is gebruik om die potensiële fitochemiese bestanddele in elke ekstrak te identifiseer en te relatief kwantifiseer.

Resultate

O.basilicum, G.glabra, I.helenium en *A.membranaceus* het die meeste van die toepaslike groepe fitobestanddele soos flavonoïde, fenole, alkaloïde, glikosides en terpenoïede op grond van die biochemiese kwalitatiewe ontleding bevat. Die water- en metanoliese ekstrakte van *O.basilicum* het omkeerbare en tydafhanklike inhibisie van CYP2B6 getoon (TAI IC_{50}s 33.35 μg/mL, 4.93 μg/mL, IC_{50}-verskuiwingsvou 1.5 vir albei ekstrakte), terwyl die metanoliese en etanoliese ekstrakte die vorming van 25-O-desasetiel-rifampisien geïnhibeer het (IC_{50}s 31 μg/mL, 8.94 μg/mL). Die metanoliese ekstrak van

O.basilicum het die hoogste TAI getoon met ’n 7.4-voudige toename in die IC_{50}. Alle ekstrakte van *I.helenium* het CYP2B6 (IC_{50}s 63 μg/mL, 89.43 μg/mL) en rifampisien-metabolisme (IC_{50}s 42.79 μg/mL, 18.58 μg/mL, 62.10 μg/mL) geïnhibeer; die waterekstrak het die hoogste TAI getoon met ’n drievoudige toename in die IC_{50}. Slegs die metanoliese en etielasetaatekstrakte van *W.somnifera* het CYP2B6 (IC_{50} 79.16 μg/mL, 57.96 μg/mL) geïnhibeer. TAI is hoofsaaklik tussen die kruie-ekstrakte en CYP2B6 waargeneem.

Die etanoliese en metanoliese ekstrakte van *A.officinalis* het CYP3A4 geïnduseer, met 48%- vouresponsverskuiwing in vergelyking met die selkontrole (geen induseerder nie). Die etanoliese ekstrak van *O.basilicum* en *G.glabra* het matige induksie van CYP3A4 en CYP2B6 veroorsaak. Die waterekstrak van *A.membranaceus* het matige en gelyke induksie van CYP3A4 en CYP2B6 (36%-vouresponstoename) getoon. Alle ekstrakte het minder as tweevoudige induksierespons (200%) getoon in vergelyking met die positiewe kontroles, rifampisien en deksametasoon.

Vername fitobestanddele waargeneem in die LC-MS/PDA-ontleding van die ekstrakte het flavonoïde, fenole, glikosides, saponiene en terpenoïede ingesluit. Relatiewe hoeveelhede van die geïdentifiseerde verbindings is bepaal in vergelyking met standaard kalibreerders, kwersetien en galliensuur (uitgedruk in mg/L ekwivalente eenhede). Fenole soos rosmariensuur (ongeveer 2298 mg/L in die waterekstrak) en kaftariensuur is gevind in *O.basilicum*-ekstrakte tesame met die flavonoïde salvigenien, rutien en isokwersetrien en ander verbindings soos linaloöl, hidroksiejasmoniese suur en eukommiol. Die waterekstrak van *I.helenium* het meestal polifenole soos chlorogeniese en kaffeoïelkiniensure bevat, terwyl die ander oplosmiddelekstrakte die seskwiterpenoïed tanasetol A, helenien (isoalantolaktoon) en makrofillielaktoon B bevat het. Die metanoliese en etielasetaat-ekstrakte van *W.somnifera* het bestaan uit witaperuvien, isopelletiërien, salvigenien, witanolides en witaferien A (ongeveer 1117 mg/L in die etielasetaatekstrak), en die ekstrakte van *A.membranaceus* het hoofsaaklik kalikosien, formononetien en astragalosiede I en IV bevat.

Die ekstrakte van *A.officinalis* het hoofsaaklik bestaan uit kiniensuur en alteaheksakosaniel-laktoon-derivate (ongeveer 3874 mg/L in die metanolekstrak), d-

galakturonsuur-monohidraat en floretien tesame met die vetsuur trihidroksie-oktadesenoësuur, en die *G.glabra*-ekstrak het hoofsaaklik bestaan uit glabridien, liwiritinapiosiede, likwiritigenien en likochalkone.

Gevolgtrekking

Daar is gevind dat *A.membranaceus*, *I.helenium*, *O.basilicum* en *W.somnifera* astragalosiede, alantolaktone, fenoliese sure en witanolides bevat wat middelmetaboliserende ensieme soos CYP2B6, CYP3A4 en die ensieme verantwoordelik vir metabolisme van rifampisien (insluitende B-esterases) kan inhibeer. *A.officinalis* en *G.glabra* kan matige induksie van CYP3A4 veroorsaak weens die hoër konsentrasie laktone en chalkone teenwoordig in hul ekstrakte. Hierdie *in vitro*-bevindinge kan relevant wees vir kliniese gebruik van hierdie kruie tesame met konvensionele medikasie wat deur hierdie ensieme gemetaboliseer is indien voldoende hepatiese konsentrasies in mense verkry word.

Die resultate van hierdie studie sal help om die beplanning en ontwerp van kliniese toetse te rig om die potensiële relevansie van KMI's onder pasiënte te bevestig.

Dedication

To my parents, Sankarankutty and Komalam,

Who always believed in me and still continue to invest into life, my dreams, and my aspirations,

To Late *Shri*. Swami Nirmalananda Giri Maharaj,

Who always inspired me to do research on herbal medicine,

And,

To Late *Dr.* David Bill Bixby Banner,

My immortalized childhood hero of a true scientist.

Acknowledgements

The work in this research study was carried out at Synexa Life Sciences, Cape Town and the Division of Clinical Pharmacology, Stellenbosch University, Cape Town and Radiant Life Sciences, Bangalore, India. I am greatly thankful to all the members in both institutions for their support and care. This study would have never happened without the financial support from the South African National Research Foundation (Indigenous Knowledge Systems NRF-IKS Grant No: 82641).

Firstly, I would like to express my heartfelt appreciation to my supervisors, Professors Bernd Rosenkranz and Patrick Bouic, whose guidance and support formed an integral part of this study. To Prof Rosenkranz, thank you for your knowledge, wisdom, and inputs in this study. I appreciate your positive attitude during many challenging circumstances. You always believed in my ability to bring this project to fulfilment, and I am always thankful to you, who selected me all the way from India to do this PhD. Prof Bouic, thank you for being my co-supervisor but foremost for always being there when I was going through lot of challenges. I so appreciate your practical input, invaluable advice, and ever willingness to lend your resources for my work. Thank you for your guidance in all aspects of my work, especially during the ELISA study as well as access to all the infrastructure and resources you provided at Synexa Life Sciences, and considering me part of the 'Synexa family'.

I am thankful to Dr. Charles Awortwe of MRC, SA for his guidance in setting up the assays at the Clinical Pharmacology Division, and his inputs to correct my mistakes during the assays. Thanks to the traditional healer of Delft, Mama Nombuso Keme and Pauline Jooste of the HOPE Cape Town team for your support with the study.

Prof Helmuth Reuter, I would like to thank you for all your support and care, which helped me so much. I also wish to thank the staff and students of the Division of Clinical Pharmacology – Prof J van Zyl – you have always been a role model to me and helped me so much since the beginning of my studies, Ms. Carine Marks, Dr. Ahmed Abulfathi, Dr. Memela Makiwane, Dr Eric Decloedt, Ms Cherylynn Wium, Mnr. Jan De Bruyn, Mnr. John Lawrence, Mnr. Henry Bester, Ms. Victoria Mathane, Ms Arina Du Plessis, Ms. Alma Van der Merwe, Gerald Fortuin, Naomi Ndahafa and Chris-Mare Agenbag - thank you for your care, support, encouragement and for all the friendly conversations that strengthened my morale during difficult moments.

A special thank you to Ms. Lejandra Hanekom for her support, encouragement, as well as assistance with any administrative work, and Dr. Lawrence Ticha for always being supportive and inspiring in this research work, and my studies since the start at Stellenbosch University; at many difficult situations your support and care was the only relief I had to forget the health issues, frustrations, and move on. And a special thanks to Paul and Charlize White – my best friends who cared for me so much, like my family.

Thanks to Mrs. Grace Bruintjies and Mr. Naweed Mullajie from the finance department in helping me procure all the research orders, and Mrs. Antonia Forbes from Leco Africa for support with HPLC columns.

A special thanks to all the staff at Synexa Life Sciences for all their help, care and support. I always felt like a family with you all – Justine Devine, Hannes van der Merwe – for accommodating me in the BACS lab and all the fun times, Nontombi Sephuhle for all the support, care and humour we shared in the lab during tough times, Rozanne Daniels for your care and friendly environment, Michael Lombard, Feroda Matthee, Daneel Johnston, Christolene Peterson, Gerhard Lamprecht, Olivia Mostert, Abeen Chethram and Alicia Chethram for being ever helpful and caring. Special thanks to the ELISA team and Matti Kimberg for guiding me with the assays and making sure I complete them on time. I would have never succeeded in the induction studies without you all. You are an awesome team!
A heartfelt thanks to Katrin Painczyk, Tenile Meyer and Lance Lucas – hadn't it been for your patience and care, I would have never managed with the logistics as well as transport of the samples.

Dr. Marietjie Stander, I would like to thank you for guiding me with the concepts on phytochemical analysis. I would like to thank Dr. Viral Patel of Radiant Life Sciences, Bangalore, India for his support and guidance in helping me complete my mRNA induction assays, training on RT-PCR equipment as well as the culturing of HepG2 cell lines; thank you especially for spending late hours and weekends with me in the lab to train me on the assays. I would like to thank the director of Radiant Life Sciences, Dr. Ashok Godavarthi for accommodating me in the cell biology lab facility.

To my family – my brothers, and friends, thank you for the love, care, encouragement and prayers. Special thanks to my wife, Chandni Porayath – our journey of research will always be

together; thanks Vinay GK for your humour and care. And last but always the first, thank you God for keeping my spirit stronger amidst all the challenges and blessing me with my guardian angels, mom and dad.

List of Publications

Full publications:

1. **S. Kumar**, P. J. Bouic and B. Rosenkranz (2017).Simultaneous HPLC Determination of Efavirenz, 8-Hydroxy Efavirenz and Neostigmine and Comparison of their Separation Using a C-18 and Biphenyl Column through Pharmacological Evaluation, *Indian J Pharm Sci*, 79 (3): 353–360, DOI: 10.4172/pharmaceutical-sciences.1000237 (Appendix 1.1).

2. **S. Kumar**, P. J. Bouic and B. Rosenkranz (2018). A Validated Stable HPLC Method for the Simultaneous Determination of Rifampicin and 25-O-desacetyl Rifampicin – Evaluation of *In vitro* Metabolism, *Acta Chroma*, pp:1-7, DOI: 10.1556/1326.2018.00361 (Appendix 1.2).

3. **S. Kumar**, N. Sephuhle, P. J. Bouic and B. Rosenkranz. HPLC/LC-MS Guided Phytochemical and *In vitro* Screening of Astragalus membranaceus (Fabaceae), and Prediction of Possible Interactions with CYP2B6, *J Herb Med* (Revised Submission #HERMED-D-17-00033R2, August 2018, *In Review*).

Abstract publications:

1. **Saneesh Kumar**, Nontombi Sephuhle, Charles Awortwe, Patrick J. Bouic, Bernd Rosenkranz (2017). HPLC/LC-MS guided phytochemical/ *in vitro* screening of *Inula Helenium* L. (Asteraceae) and *Althaea officinalis* L. (Malvaceae), and prediction of possible cytochrome P450 interactions, *IntJ Pharm Pharm Sci*, [S.I., Sep. 2017], p. 13. (Appendix 1.2).

2. **Saneesh Kumar**, Nontombi Sephuhle, Charles Awortwe, Patrick J. Bouic, Bernd Rosenkranz (2015).HPLC/LC-MS guided phytochemical screening of *Astragalus membranaceus* and predictions of possible cytochrome P450 interactions, *Int J Pharm Pharm Sci*, [S.I., Feb. 2015], p. 53. (Appendix 1.3).

Manuscripts in preparation:

1. **S. Kumar**, P. J. Bouic and B. Rosenkranz. Investigation of CYP2B6 interactions of *Withania somnifera* (L.) in human liver microsomes and HepG2 cells.

2. **S. Kumar**, P. J. Bouic and B. Rosenkranz. *In vitro* assessment of the interaction potential of *Ocimum basilicum* (L.) on CYP2B6, 3A4 and rifampicin metabolism using human liver microsomes and mRNA gene expression.

List of conferences attended

1. **Saneesh Kumar**, C. Awortwe, P.J. Bouic, B. Rosenkranz: HPLC/LC-MS guided phytochemical/ *in vitro* screening of African herbal medicines and predictions of possible cytochrome P450 interactions.
   INNOPHARM 2, Second International Conference on Novel Frontiers in Pharmaceutical and Health Sciences, Bhopal (India) (10 – 11 February 2017, abstract published in the International Journal of Pharmacy and Pharmaceutical Sciences).

2. **Saneesh Kumar**, Patrick J Bouic, Bernd Rosenkranz: Pharmacokinetics of herb-drug interactions involving African traditional medicines – fingerprint analysis and *in vitro* metabolism study.
   UNISA DST/NRF IKS INTERFACE 4 "Towards Science and Technology of Humility in South Africa", Khoroni, Venda, SA (03 – 07 December 2016, was selected as one of the best presentations of the conference).

3. **Saneesh Kumar**, Nontombi Sephule, C. Awortwe, P.J. Bouic, B. Rosenkranz: HPLC/LC-MS guided phytochemical screening of *Astragalus membranaceus* and predictions of possible cytochrome P450 interactions.
   INNOPHARM 1, First International Conference on Novel Frontiers in Pharmaceutical and Health Sciences, Bhopal (India) (10 – 11 October 2015, abstract published in the International Journal of Pharmacy and Pharmaceutical Sciences).

4. **Saneesh Kumar**, Patrick J Bouic, Bernd Rosenkranz: IKS Bio Economy: Effect of traditional medicines on drug pharmacokinetics.
   UNISA DST/NRF IKS INTERFACE 3 "Building Excellence in Indigenous Knowledge Systems as a Science", Pretoria, SA (27 February – 1 March 2015).

Abbreviations

[I]	Inhibitor concentration
[S]	Substrate concentration
25ODESRIF	25-O-desacetyl rifampicin
Ab	Antibody
ADMET	Absorption, distribution, metabolism, and excretion and Toxicity
AGE	Agarose gel electrophoresis
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immunodeficiency syndrome
AP	Alkaline phosphatase
ART	Antiretroviral therapy
ARVs	Antiretroviral drugs
AUC	Area under the curve
bDNA	Branched DNA
BSA	Bovine serum albumin
CAM	Complementary and alternative medicines
CAR	Constitutive androstane receptor
cART	Combination Antiretroviral Therapy
CES	Carboxylesterase
CL_{int}	Intrinsic clearance
CMV	Cytomegalovirus
CTC_{50}	Concentration of the extract that result in 50% cytotoxicity of HepG2 cells
CV	Coefficient of Variation
CYP	Cytochrome P 450
DR-TB	Drug-resistant tuberculosis
E	Enzyme
EFV	Efavirenz
ELISA	Enzyme–linked immunoassay
ESCI	Electrospray ionisation and chemical ionization
ESI	Electrospray ionization
DDI	Drug-drug interactions
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography
GIT	Gastrointestinal tract
HDI	Herb-drug interaction
HIV	Human immunodeficiency virus
HLM	Human liver microsomes
HPLC – PDA	High performance liquid chromatography – Photodiode array detector
HPLC – VWD	High performance liquid chromatography – Variable wavelength detector
HRP	Horseradish peroxidase
I.S.	Internal standard
IC_{50}	Concentration of inhibitor that results in 50% inhibition of enzyme activity
IgG	Immunoglobulin G

K_i	Inhibition constant (reversible inhibition)
K_m	Michaelis-Menten constant
K_s	Substrate affinity for an enzyme
LC-MS	Liquid chromatography–mass spectrometry
LLOD	Lower limits of detection
LLOQ	Lower Limits of quantification
LOD	Limits of detection
LOQ	Limits of quantification
MALDI – TOF	Matrix-assisted laser desorption/ionization – Time of flight
MBI	Mechanism-based inhibition
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NELF	Nelfinavir
NEO	Neostigmine
NMR	Nuclear magnetic resonance spectroscopy
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
OATP	Organic Anion Transporting Polypeptide
OD	Optical density
P	Product
PAb	Primary antibody
PBREM	Phenobarbital-responsive enhancer module
PBS	Phosphate buffered saline
PDA	Photo diode array
P-gp	P-glycoprotein
PXR	Pregnane X receptor
Q-TOF	Quadrupole time-of-flight
QqQ	Triple quadrupole
RIF	Rifampicin
RT	Retention time
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SEM	Standard error of the mean
TAT2	Telomerase activator 2
TCM	Traditional Chinese medicine
TDI	Time dependent inhibition
THMs	Traditional herbal medicines
THPs	Traditional health practitioners
TICL	Ticlopidine
TK	Thymidine kinase
TMB	3,3’,5,5’-Tetramethylbenzidine
THP	Traditional health practitioner
UGT	Uridine diphosphate glucuronosyl transferase
UV-Vis	Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry
V_{max}	Maximal velocity or maximum rate of reaction
WHO	World Health Organization

UNITS

bp	Base pair
mg	Microgram
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar
g	Gram
h	Hour
kb	Kilobase
mg	Milligram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
min	Minute
mL	Millilitre
mL min^{-1}	Millilitre per minute
mm	Millimetre
mM	Millimolar
m/z	Mass-to-charge ratio
ng	Nanogram
pg	Picogram
rpm	Revolutions per minute
U/ml	Units per millilitre
$^{w}/_{w}$	Weight per weight

Table of Contents

Chapter 1
INTRODUCTION

1.1 Overview of drug metabolism

The human liver is the principal site of drug metabolism, where a drug is chemically converted to a metabolite through oxidation, reduction, conjugation and hydrolysis, prior to its elimination from the body. The study of this biotransformation is critical to understand the clinical profile of a drug, the pharmacokinetics and toxicology of its metabolites, determination of the dosage regimens and its potential to cause interactions in combination with other drugs. The human body is constantly exposed to 'xenobiotics' or foreign substances such as chemicals from the food, air, water as well as drugs or herbal supplements for treating diseases, and has various enzymes to eliminate these substances.

The xenobiotic metabolism occurs in two phases. Phase I involves oxidation, reduction and hydrolysis of the lipid-soluble drug. Phase II involves reactions that are synthetic in nature, and the conjugation with an endogenous substance such as glucuronic acid, sulfate or glycine; whereby polar metabolites are formed which are excreted by the kidney (urine) and liver (bile) (White, 2012). Cytochrome P450 (CYP), a microsomal group of isoenzymes catalyse the oxidation of xenobiotics in phase I. These enzymes form the basis of this study.

1.2 Cytochrome P450 enzymes

CYPs are the major enzymes involved in drug metabolism, accounting for about 75% of the total metabolism of the drugs within the human body (Guengerich, 2008; Williams et al., 2005). Most drugs undergo deactivation by CYPs, either directly or by facilitated excretion from the body, although some substances are bioactivated by CYPs by formation of active metabolites. Human CYPs are primarily located either in the mitochondrial inner membrane or the endoplasmic reticulum of cells. Many endogenous and exogenous chemical substances are primarily metabolised by these CYPs. Some CYPs such as CYP19 (aromatase) metabolize only one substrate (or very few), while others may metabolize multiple substrates. These characteristics give the CYPs a very important role in the metabolism of medicine. CYPs play critical roles in the synthesis and breakdown of hormones, including estrogen and testosterone synthesis and metabolism as well as cholesterol synthesis, and the metabolism of vitamin D (Salphati and Benet, 1998; Guengerich, 2008; Bjorkman, 2005).

Potentially toxic compounds including products of endogenous metabolism (such as bilirubin) and drugs are also metabolised by the CYPs, primarily in the liver. Drugs are predominantly biotransformed by a limited subset of CYPs, namely CYP1A2, CYP2A6, CYP2B6, CYP2Cs (principally CYP2C8, CYP2C9 and CYP2C19), CYP2D6, CYP2E1 and CYP3A4 (Gomez-Lechon et al., 2004; DiPiro, 1999; Cupp and Tracy, 1998). These CYPs are responsible for more than 50% of the total drug metabolism (Wilkinson, 2005). Recent studies revised the CYP distribution 'pie' chart to include CYP4F enzymes to the total contribution of all the hepatic P450 enzymes, as illustrated in Fig. 1 (Michaels and Wang, 2014).

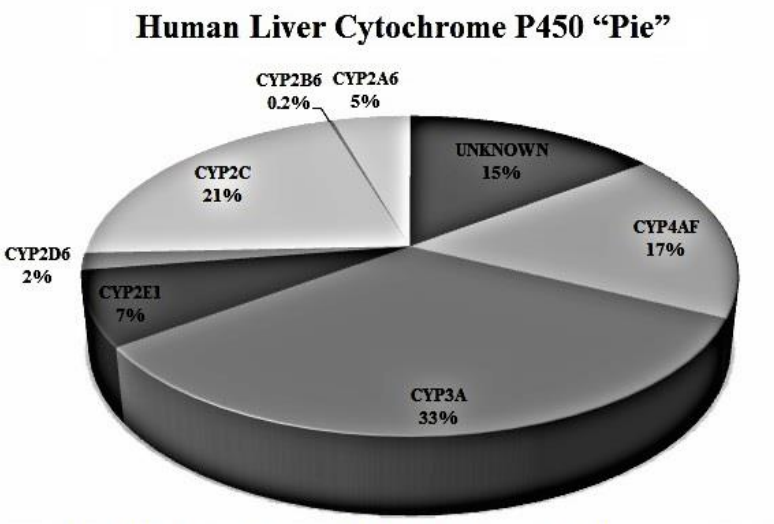


Fig.1 The Cytochrome P450 *pie* chart, with the percentage contribution of individual CYPs, to the total hepatic P450 enzymes (Michaels and Wang, 2014)

Inhibition or induction of these enzymes can result in altered clearance and pharmacokinetics (PK) of drugs leading to very high or low concentrations and therefore to adverse effects or lack of efficacy. This is particularly important considering that the aging population is often on multiple drugs (polypharmacy) for various ailments. Therefore, evaluation of drug interactions is part of the assessment of drug efficacy and safety.

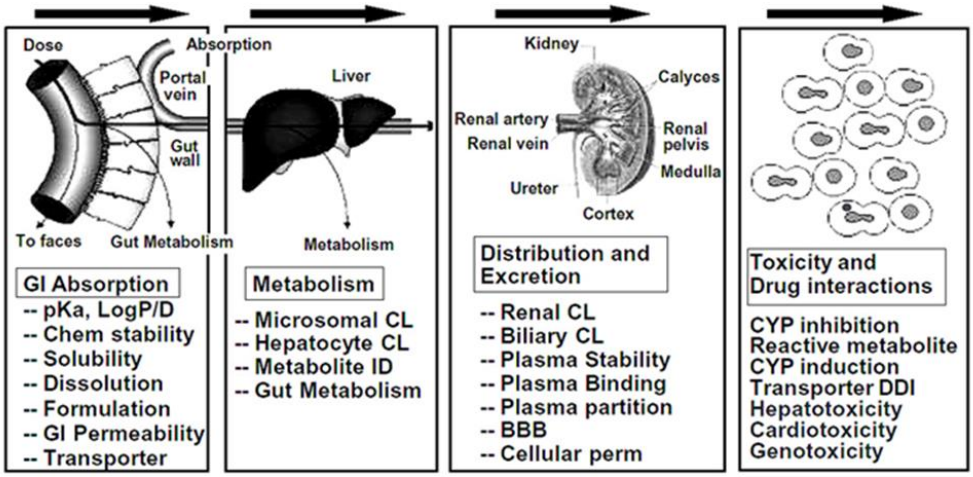


Fig. 2 ADMET profiling paradigm in drug discovery and development. The potential of the drug to inhibit/induce CYP-mediated metabolism attributes to probable drug-drug interactions (DDI) and toxicity (Zhang and Surapaneni, 2012).

***** GI – gastrointestinal, CL – clearance***

The screening of absorption, distribution, metabolism, excretion and toxicity (ADMET) drug properties during a drug discovery and development process, as well as understanding drug interactions with the CYPs is critical (Fig. 2), and has the potential to increase the chances for identification of a new drug candidate within a shorter time frame (Zhang and Surapaneni, 2012).

CYP3A4 is the most prevalent in humans, and the most important enzyme in terms of drug biotransformation. CYP3A4 isoenzyme is involved in many clinically significant drug-drug interactions (DDI). Studies involving CYP3A4 and DDI/HDI are becoming an integral part of drug research (Ogu and Maxa, 2000). Many studies have recorded the interaction of herbal medicines prominently with CYP3A4, along with other CYPs, whereby the CYP3A4-herb interaction analysis is an evaluation model of high importance (Wanwimolruk et al., 2014).

CYP2B6 is involved in the metabolism of clinically important drugs. The discovery of extensive variations of the CYP2B6 gene (the most common being *CYP2B6*6*), the potential effects of ethnic variations on its expression as well as the emerging evidence for its clinical relevance, have demonstrated the important role of this enzyme (Desta and Flockhart, 2017). Most of the HDI evaluations have been performed using CYP2D6, CYP1A2, CYP2C19, CYP2C9, CYP3A4, and CYP2E1, but very little information is available on how herbal medicines affect the expression of CYP2B6. Considering the high prevalence of HIV/AIDS across Africa, the wide-spread use of efavirenz in treatment, and the variation of CYP2B6 expression on efavirenz metabolism, one of the main objectives of this study was to assess the influence of selected herbal medicines on the activity of CYP2B6, including its functional characterization.

1.1.1 Cytochrome P450 2B6

The cytochrome P450 (CYP) enzyme CYP2B6 is one among 12 human CYPs, primarily involved in the metabolism of drugs and other xenobiotics and constitutes less than 1% of the total hepatic CYP content (Zanger et al., 2007). This highly polymorphic enzyme has upto 38 reported allelic variants (PharmVar, 2017). Therapeutically important drugs metabolized primarily by CYP2B6 include the non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz (Ward et al., 2003; Desta et al., 2007). Besides efavirenz, drugs such as artemisinin, bupropion, ketamine, methadone and cyclophosphamide (~8% of those on the market) undergo metabolism via the CYP2B6 pathway.

CYP2B6 genotype is the most important genetic factor influencing plasma efavirenz concentrations. Human CYP2B6 is strongly inducible by several drugs including phenytoin and phenobarbital. Enzyme induction involves phenobarbital-responsive enhancer module (PBREM,-1.7 kb) and a distal xenobiotics-responsive enhancer module (XREM, -8.5 kb). The pregnane X receptor (PXR, NR1I2) and/or constitutive androstane receptor (CAR, NR1I3) bind to this module to mediate an increase in transcription (Sueyoshi et al., 1999; Wang et al., 2003; Faucette et al., 2004). The variants of CYP2B6 can affect transcriptional regulation, splicing, mRNA and protein expression, and catalytic activity. These polymorphisms are clinically relevant for HIV-infected patients treated with the reverse transcriptase inhibitor efavirenz, along with other drug substrates of CYP2B6 (Zanger and Klein, 2013). Previous studies have shown the induction of CYP2B6 by herbal medicine (Fan et al., 2009) as well as the inhibitory effect of African herbal medicines on CYP2B6 (Thomford et al., 2016).This study focuses on CYP2B6 because of its primary involvement in the metabolism of efavirenz, and the impact of herbal medicines that are co-administered with efavirenz in HIV patients.

1.1.1.1 Efavirenz metabolism

Efavirenz containing combined antiretroviral therapy (ART) is the first line treatment (Manosuthi et al., 2016) in Sub-Saharan Africa. Efavirenz is primarily metabolized to 8-Hydroxy efavirenz mainly by CYP2B6 and to a lesser extent by CYP3A and, to 7-Hydroxy efavirenz by CYP2A6. 8-Hydroxyefavirenz is metabolized to 8,14-dihydroxy efavirenz by CYP2B6 (Ward et al., 2003). Ethnicity, CYP2B6*6 and ABCB1 c.4036A/G have been shown to be significant predictors of efavirenz pharmacokinetics in HIV/AIDS patients in Africa (Ngaimisi et al., 2013).

1.1.2 Cytochrome P450 3A

Fifty–60% of all CYP drug metabolism is mediated by the CYP3A subfamily of enzymes (Slaughter and Edwards, 1995), which comprises of CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (Daly, 2006). Among these, CYP3A4 forms the major portion of CYP3A protein present in the human liver and up to 90-fold variations may be observed in its catalytic activity. Subjects carrying defective alleles of CYP3A4 may be predisposed to health conditions such as prostate cancer, type 2 diabetes mellitus and estrogen receptor negative breast cancer (Lamba et al., 2002). CYP3A5 is polymorphically expressed in about 10-30% human livers, and represents upto 50%of the total hepatic CYP3A in such scenarios (Roy et al., 2005; Daly, 2006; Lamba et al., 2002). Its amino acid sequence (85%) is identical to CYP3A4;

however it varies in its catalytic activity and regioselectivity towards substrates (Daly, 2006). The CYP3A5 gene contributes to inter-individual and inter-racial variation of CYP3A mediated metabolism of drugs, including anticancer drugs (irinotecan, docetaxel, vincristine), antimalarial (mefloquine, artemether), antipsychotics (olanzapine), antiestrogen (tamoxifen), immunomodulators (tacrolimus, cyclosporine), antibiotics (clarithromycin), antihistamines (chlorpheniramine, terfenadine, astemizole), antihypertensives (verapamil, nifedipine, amlodipine, felodipine), antivirals (indinavir, nelfinavir, ritonavir, saquinavir), steroids (testosterone, estradiol, progesterone and androstenedione) (Shimada & Guengerich, 1989; Flockhart, 2007) and carcinogens (Gene: cytochrome P450, 2017).

Cytochromes belonging to the CYP3A subfamily have large active sites that bind to multiple substrates that are structurally different. Kinetic models have been designed on the assumption that CYP3A4 contains two distinct and independent substrate binding domains within the active site, resulting in the metabolism of two different substrates coexisting at the active site. The resultant velocity equations were used to derive kinetic parameters involved in the substrate-enzyme reactions and the nature of metabolic interaction between the two substrates was described using the kinetic constants (Shou et al., 2001).

CYP3A4 can be induced more than the other CYPs (Dogra et al., 1998; Denison and Whitlock, 1995; Waxman and Azaroff, 1992). CYP3A4 is induced by rifampicin, HIV antiretroviral drugs such as efavirenz and nevirapine, carbamazepine, dexamethasone, glucocorticoids, phenobarbital, phenytoin, sulfadimidine and St. John's wort (Flockhart, 2007). Inhibitors of CYP3A4 include nelfinavir, ketoconazole, grapefruit juice, quinine, erythromycin, itraconazole, valproic acid, telithromycin, and verapamil (Flockhart, 2007). The metabolism pathway catalysed by CYP3A4 forms a major route for elimination of many drugs, and the induction of its expression is often implicated in clinically relevant drug-drug interactions. Rifampicin, a potent inducer of CYP3A4 activity (Hellum et al., 2007; Gurley et al., 2006; Erickson et al., 1999), is commonly used as a positive control to investigate and evaluate the induction potential of test compounds on CYP3A4. CYP3A4 is a key enzyme related to DDIs occurring in TB treatment, particularly drug-resistant TB, and co-infection with TB/HIV (Kwara et al., 2010).

1.1.3 Rifampicin metabolism and esterases

Rifampicin is part of the major regimen for the treatment of active TB disease, including HIV-infected patients (Department of Health and Human Services, 2016; British HIV Association,

2011). Rifampicin induces CYP3A4 mRNA in liver HepG2 and blood mononuclear cells (Glaeser et al., 2005; Gashaw et al., 2003). Rifampicin induced gut and liver (hepatic) CYP3A4, and autoinduces its own metabolism (Kliewer and Willson, 2002). Rifampicin is metabolized to 25-O-desacetyl rifampicin, via deacetylation by esterases or by enzymes present in microsomal cells (Jamis-Dow et al., 1997). The des-acetyl derivative is eliminated in bile. Some of its other metabolites include 3-formyl rifamycin, rifampicin N-oxide, and rifampicin quinone (Mohan et al., 2003). P-glycoprotein (P-gp) transporter induction along with B-esterases, are involved in disposition of rifampicin (Staudinger et al., 2010).

B-esterases are enzymes that hydrolyse carboxyl esters and exhibit very broad substrate specificity. Acetylcholinesterase, human carboxylesterase (CES), and butyrylcholinesterase belong to the B-esterase family, CES2 being the most important enzyme with the broadest substrate specificity. Variations in the promoter region of CES2 affect the gene expression, and alter the metabolism of rifampicin (Song et al., 2013).

Another study demonstrated that decreased expression of CYP3A4 contributed to increased oral absorption of rifampicin, indicating the possible involvement of CYP3A4 in pre-systemic metabolism of rifampicin (Prakash et al., 2003; Venkatesan, 1989).

Rifampicin-induced CYP3A4 activation and expression models have been used for predicting the pharmacokinetics of CYP3A4 substrate drugs as well as clinically relevant DDI (Yamashita et al., 2013). Most of the *in vitro* HDI studies investigate the role of rifampicin as an inducer of CYP3A4 along with other drugs and herbal extracts; less information is available on the effect of herbal medicines on the metabolism pathway of rifampicin to 25-O-desacetyl rifampicin. *In vitro* studies have not explored the use of rifampicin as a substrate for HLM assays, to analyse the effect of herbal medicines on its pathway by measuring the enzyme kinetics of 25-O-desacetyl rifampicin.

In this study the effects of the selected herbs on the rifampicin metabolism pathway was investigated to understand the changes in its pharmacokinetics associated with 25-O-desacetyl rifampicin. The potential of the herbs tested to inhibit this pathway was ranked based on their IC_{50} values.

1.3 HIV/AIDS and tuberculosis

Drug interactions pose an increasingly complex challenge for clinicians treating HIV infected patients. Current treatment guidelines recommend the use of a combination of at least three anti-retroviral drugs for the treatment of HIV-infected patients (Combination Antiretroviral Therapy, cART). In addition to medications to treat HIV infection, patients often receive therapy for concomitant conditions or for prophylaxis of opportunistic infections. Due to the amount of drugs received by an HIV-infected patient, clinicians are often forced to rely on clinical judgment and predict drug interactions without having supporting data (HIV Clinical Resource, 2010).

Drug interactions involving metabolism are the most common, and their clinical relevance is often difficult to predict. Most drugs used in cART, especially NNRTIs and protease inhibitors, are metabolized via the CYP system. NNRTI-based cART remains a first-line regimen for the treatment of HIV-infected patients with TB in resource-limited settings. Efavirenz is a preferred NNRTI with favourable treatment outcomes (Meintjes et al., 2017; Manosuthi et al., 2016).

TB is an opportunistic infection with a higher prevalence in immune compromised patients, such as HIV-infected individuals. Medications used to treat TB can have significant interactions with other drugs. The interaction that is clinically most relevant is that of the enzyme induction associated with rifampicin, a first-line agent used in combination with isoniazid for the treatment of TB infection (Kerantzas et al., 2017; Kreek et al., 1976). The standard treatment of drug-susceptible TB comprises of a 2-month initial intensive phase comprising of rifampicin, pyrazinamide, isoniazid and ethambutol, followed by 4 months of continuation phase of rifampicin and isoniazid. cART should be initiated in all HIV-infected patients with TB, within the first 8 weeks of starting antituberculous treatment and within the first 2 weeks for patients with CD4 cell counts <50 cells/mm3 (WHO TB guidelines, 2017; Manosuthi et al., 2016). Isoniazid, a CYP3A4 inhibitor (Wen et al., 2002) when used along with rifampicin may also induce various CYP isoenzymes leading to the formation of acetyl-isoniazid from isoniazid. The latter is further converted to monoacetyl hydrazine and catalysed by CYPs to form other hepato-toxic compounds, thereby increasing isoniazid toxicity (Chen and Raymond, 2006).

Selection of the appropriate drugs prescribed for HIV/AIDS and TB, is critical. The treatment guidelines take into account the effect of each drug on the CYP system as shown in Table [1] below.

Table [1] Drugs and CYP Interactions (Lee et al., 2014; FDA Clinical Pharmacology Guidelines, 2012; HIV Clinical Resource, 2010; Arbex et al., 2010; Kivisto et al., 1995)

DRUG	EFFECT ON THE CYP SYSTEM
HIV/AIDS TREATMENTS	
Non-Nucleoside Reverse Transcriptase Inhibitors	
Delavirdine (DLV)	CYP3A4 inhibitor
Efavirenz (EFV)	CYP3A4 inducer (inhibitor *in vitro*)
Etravirine (ETR)	CYP3A4 Inducer, CYP2C9 and CYP2C19 inhibitor
Nevirapine (NVP)	CYP3A4 inducer
Protease Inhibitors	
Amprenavir (APV), Fosamprenavir (FPV)	CYP3A4 inhibitor/inducer
Atazanavir (ATZ)	CYP3A4 inhibitor, CYP1A2, CYP2C9 inhibitor
Darunavir/r (DRV/r)	CYP3A4 inhibitor
Indinavir (IDV)	CYP3A4 inhibitor
Lopinavir/ritonavir (LPV/r)	CYP3A4 inhibitor CYP2D6 inhibitor (CYP3A4 inhibition > CYP2D6) CYP3A4 and CYP1A2 inducer
Protease Inhibitors	
Amprenavir (APV), Fosamprenavir (FPV)	CYP3A4 inhibitor/inducer
Atazanavir (ATZ)	CYP3A4, CYP1A2, CYP2C9 inhibitor
Darunavir/r (DRV/r)	CYP3A4 inhibitor
Indinavir (IDV)	CYP3A4 inhibitor
Lopinavir/ritonavir (LPV/r)	CYP3A4, CYP2D6 inhibitor; (CYP3A4 inhibition > CYP2D6); CYP3A4 and CYP1A2 inducer
Nelfinavir (NFV)	CYP3A4 inhibitor/inducer
Ritonavir (RTV)	CYP3A4, CYP2D6 inhibitor; (3A4 inhibition >CYP2D6); CYP3A4 and CYP1A2 inducer
Saquinavir (SQV)	Weak CYP3A4 inhibitor
Tipranavir/ritonavir (TPV/r)	CYP3A4 inhibitor Potent P-gp inducer
TUBERCULOSIS TREATMENTS	
Pyrazinamide	No significant CYP interactions
Rifampicin	CYP3A4 and CYP2C9 Inducer
Isoniazid	Inhibitor on CYP2C9, CYP2C19, and CYP2E1, but its effect on the CYP3A family is minimal.
Ethambutol	Strong inhibitory potential against CYP1A2 and CYP2E1, moderate against CYP2C19 and CYP2D6 and weak against CYP2A6, CYP2C9 and CYP3A4

1.4 Inhibition of CYP enzyme Activity

Clinical DDI or HDI involving inhibition or time-dependent inactivation of the CYP enzymes can result in clinically relevant side effects resulting from reduced clearance/increased exposure of the drug being affected, and diminished clinical efficacy with increased dosage requirements. Enzyme inhibition is classified as reversible or irreversible. In the case of reversible inhibition, the inhibitor binds non-covalently; irreversible inhibitors commonly bind with the enzyme through the formation of covalent bonds or react with residues/ by-products involved in the catalysis and alter them chemically. Since reversible inhibitors do not react with the enzyme and form any chemical bonds, they are formed rapidly and can be easily separated, thus the enzyme-inhibitor complex gets dissociated quickly in contrast to irreversible inhibition (Alsanosi et al., 2014).

1.4.1 Reversible inhibition

In reversible inhibition (further subdivided into competitive, non-competitive, uncompetitive, and mixed types) the enzyme activity is fully restored when the inhibitor is removed from the system in which the enzyme functions. In reversible inhibition, there exists equilibrium between the inhibitor, I, and the enzyme, E [equation (1.4.1)]:

$$E+I \rightleftharpoons EI \qquad (1.4.1)$$

The equilibrium constant (K_i, the inhibitor constant) for the dissociation of the enzyme–inhibitor complex, is given by equation (1.4.2).

$$K_i=[E][I][EI] \qquad (1.4.2)$$

Thus, K_i is a measure of the affinity of the inhibitor for an enzyme, similar to the constant K_s, which is a measure of the affinity of a substrate for an enzyme (Ochs, 2000).

1.4.1.1 Competitive inhibition

In competitive inhibition, the inhibitor is commonly a structural analogue of the substrate S and competes with it to bind at the active site thereby forming the product of the reaction P (Ochs, 2000).

Thus, two reactions are possible:

$$E+S \rightleftharpoons ES \rightarrow E+P \qquad (1.4.3)$$

$$E+I \rightleftharpoons EI \qquad (1.4.4)$$

The metabolic rate of the reaction (v) can be expressed as:

$$v = V_{max}\,[S]/\, K_m(1+[I]/K_i) + [S] \qquad (1.4.5)$$

Where, V_{max} is the maximum velocity of the reaction, and K_m is the Michaelis-Menten constant- the substrate concentration at which the reaction rate is half of V_{max} (Nelson and Cox, 2008).

1.4.1.2 Non-competitive inhibition

In non-competitive inhibition, the inhibitor binds to the enzyme at a site different from the substrate binding site, and has no structural resemblance to the substrate. There is no competition between the inhibitor and the substrate, and increasing substrate concentration cannot overcome the inhibition (Ochs, 2000). The inhibitor binds either to the enzyme–substrate complex or a free enzyme; in both cases, the complex formed is catalytically inactive. Non-competitive reactions are shown in equations (1.4.6) and (1.4.7).

$$E+I \leftrightarrow EI \text{ (inactive)} \quad (1.4.6)$$

$$ES+I \leftrightarrow ESI \text{ (inactive)} \quad (1.4.7)$$

The value of V_{max} is reduced by the inhibitor because the concentration of active enzyme is reduced; K_m is unaffected.

The metabolic rate of the reaction can be expressed as:

$$v = \{V_{max}/ (1+[I]/K_i)\} [S]/K_m + [S] \quad (1.4.8)$$

1.4.1.3 Uncompetitive inhibition

In uncompetitive inhibition, the inhibitor binds to the enzyme once the substrate binding is complete, and not alone. Consequently, the likelihood of uncompetitive inhibition occurs in conditions where the concentrations of both substrate and enzyme are high.

The metabolic rate of the reaction can be expressed as:

$$v = \{V_{max}/ (1+[I]/K_i)\} [S]/K_m/(1+[I]/K_i) + [S] \quad (1.4.9)$$

A special case of uncompetitive inhibition is substrate inhibition, where the substrate itself binds to the enzyme at high concentrations, and blocks its activity.

1.4.1.4 Mixed inhibition

Mixed inhibition is considered a ‘mixture’ of competitive inhibition and uncompetitive inhibition. The inhibitor can bind to the enzyme alone, before the substrate binds (such as in competitive inhibition), or can bind to the enzyme substrate complex formed (such as in uncompetitive inhibition) (Ramsay and Tipton, 2017; Ochs, 2000). However, the inhibitor has a greater affinity for one of the two states, and not both.

The metabolic rate of the reaction can be expressed as:

$$v = V_{max}/ (1+[I]/K_i) + (1 + K_s /[S]) \times (1 + [I]/K_i) \quad (1.4.10)$$

1.4.2 Irreversible inhibition

In irreversible inhibition, also referred to as mechanism-based inhibition (MBI) or time-dependent inhibition (TDI), the rate of metabolic recovery is similar to reversible inhibition, and dependent upon the production of new enzyme, rather than the dissociation of the enzyme-inhibitor complex, and elimination of the inhibitor.

In TDI, the potency of the inhibitor increases upon prolonged exposure to the enzyme during the pre-incubation period, thereby reducing the enzyme activity. Metabolites that possess stronger inhibitory activity than the parent molecule are formed during such reactions, which bind irreversibly to the enzyme and inactivate it.

MBI can further be divided into quasi-irreversible and true irreversible inhibition. In quasi-irreversible inhibition, the metabolite of the reaction interacts with the ferrous structure of the CYP heme, and forms a metabolic intermediate complex that makes the enzyme inactive. In true irreversible inhibition, the substrate forms strong covalent bonds with the enzyme and cannot be dissociated (Tihanyi and Vastag, 2011).

1.5 Traditional medicines

Traditional medicines which include herbal remedies, homeopathy, oriental medicine and acupuncture, and spiritual therapies have been used for millennia to treat acute and chronic diseases. At least 28,000 plant species are currently recorded as being of medicinal use, although less than 16% of the species used in plant-based medicines are cited in a medicinal regulatory publication (SOTWP, 2017). In many regions of the world, people rely on traditional herbal medicines (THMs) for their primary healthcare (Tapsell et al., 2006). In Germany, it is estimated that 90% of the population use herbal medicines (WHO TMS, 2014; Payyappallimana and Subramanian, 2015; Ong et al., 2005). Herbal medications have been used in India, African countries, China, Central and South America, and Europe for centuries. The Indian traditional medical system called *Ayurveda* (*Sanskrit*: *Ayur* means ‘longevity’ or ‘life’ and *Veda* means ‘science’) dates back to over 5000 years and the practices that are derived from *Ayurvedic* medicine are regarded as part of complementary and alternative medicines (CAM) (NCCAM-NIH, 2006), and along with Siddha Medicine and Traditional Chinese medicine (TCM), forms the basis for systems medicine. In December 2016, the Chinese government recognised improved scientific understanding of the TCM and its value in treating chronic conditions by

announcing their aim to integrate the same into their healthcare system by 2020 (Xinhuanet, 2016).

Traditional African medicine is a holistic discipline involving indigenous herbs and African spirituality, typically involving herbalists, spiritual healers, diviners and midwives. Practitioners of traditional African medicine claim to be able to cure diverse health conditions such as HIV-AIDS, TB, cancers, psychiatric disorders, high blood pressure and cholera (Helwig, 2005). In recent years, the herbal remedies and treatment methods used in traditional African medicine have gained more appreciation from researchers in medicine and technology. Developing countries have started assessing the high costs involved in the establishment of modern health care systems with new and advanced technologies (Mahomoodally, 2013). Due to this reason, many African countries have recently expressed interest in the integration of traditional African medicine into the continent's national health care systems (Ubani, 2011). Organizations like the National Research Foundation of South Africa, have allocated funds for research on promoting indigenous knowledge systems in the country.

The traditional health practitioners (THPs) use a wide variety of treatments ranging from ‘magic’ and spiritual healing, to biomedical methods such as fasting and dieting, herbal therapies, bathing, massage, and other surgical procedures (Conserve Africa, 2002). Conditions such as migraine, abscesses, pleurisy and cough are often cured using the method of "bleed-cupping" and the application of herbal ointments followed by herbal drugs (Mehta and Dhapte, 2015). In some African cultures, hot herbal ointment is rubbed across the patient's eyelids to cure migraines and headaches. Herbal mixtures are often used as cures for malaria, which involves steam inhalations of herbal concoctions (Onwuanibe, 1979).

In tropical Africa, over 4000 plants are popularly used as medicinal plants (Ubani, 2011; Stanley, 2004). Medicinal plants are used to treat many diseases, illnesses and other health conditions, the uses and effects of which are of growing interest to Western societies and the pharmaceutical researchers. Plants such as *Siphonochilus aethiopicus* (African ginger), *Lessertia frutescens* (Cancer bush), *Aloe ferox*, *Hypoxis hemerocallidea* (African potato), *Harpagophytum procumbens* (Devil’s claw), *Tulbaghia violacea* (Wild garlic), etc., are some of the plants classified as medicinal in Southern Africa (Directorate Plant Pr., 2013). Certain pharmacological and toxicological effects of plants such as *Lessertia* and *Hypoxis* have been identified when used for patients with HIV/AIDS (Mills et al., 2005).

Some of the commonly used medicinal plants in Cameroon, Tanzania, Madagascar and Malawi include *Prunus africana* (Pygeum), *Securidaca longipedunculata*, *Justicia flava*, *Lessertia frutescens*, *Agaura salicifolia*, *Turraea holstii*, *Jatropha multifida*, *Sterculia africana*, *Spirostachys africana*, *Turraeanthus africanus*, *Antiaris africana* and *Albizia adianthifolia* (Ntie-Kang et al., 2013; van den Bout-van den Beukel et al., 2008). *Acacia senegal* (Senegal gum), *Artemisia herba-alba*, *Aspalathus linearis* (rooibos), *Centella asiatica*, *Catharanthus roseus*, *Momordica charantia* (bitter melon), *Cyclopia genistoides*, *Momordica charantia* and *Pelargonium sidoides* are among the other commonly used medicinal plants that form part of African herbal pharmacopeia with commercial importance (Mahomoodally, 2013).

1.6 Herb-drug interactions

Today herbs are widely used in teas, vitamins and natural supplements. Besides the potential benefits of herbal medicine, it is important to recognize possible HDI, which might have pharmacological or toxicological effects. Overdose of certain herbs or conventional drugs can be toxic. Synergistic therapeutic effects may lead to adverse complications in the dosing of long-term medications – e.g., herbs traditionally used to decrease glucose concentrations in diabetes (Bailey and Day, 1989) could theoretically precipitate hypoglycaemia if taken in combination with conventional drugs (Bailey et al., 1989). Herbal medicines are complex mixtures of more than one active ingredient (phytocompounds). The multitude of such phytocompounds which are pharmacologically active increases the likelihood of interactions with other conventional drugs. Hence, the chances of HDI are theoretically higher than drug–drug interactions, because synthetic drugs usually comprise of a single molecule or a few chemical entities.

Various classes of chemical compounds are present in plants, which include flavonoids, phenols, saponins, glycosides, alkaloids, terpenoids, and coumarins. Some of these compounds are potentially capable of interacting with the CYPs, and altering their expression and activity. Previous research has reported particular class of phytocompounds including the structure of some of the molecules (Fig. 3) responsible for causing HDI. This model connects the phytochemical constituents of the herb to its effect (previously reported) on the CYPs.

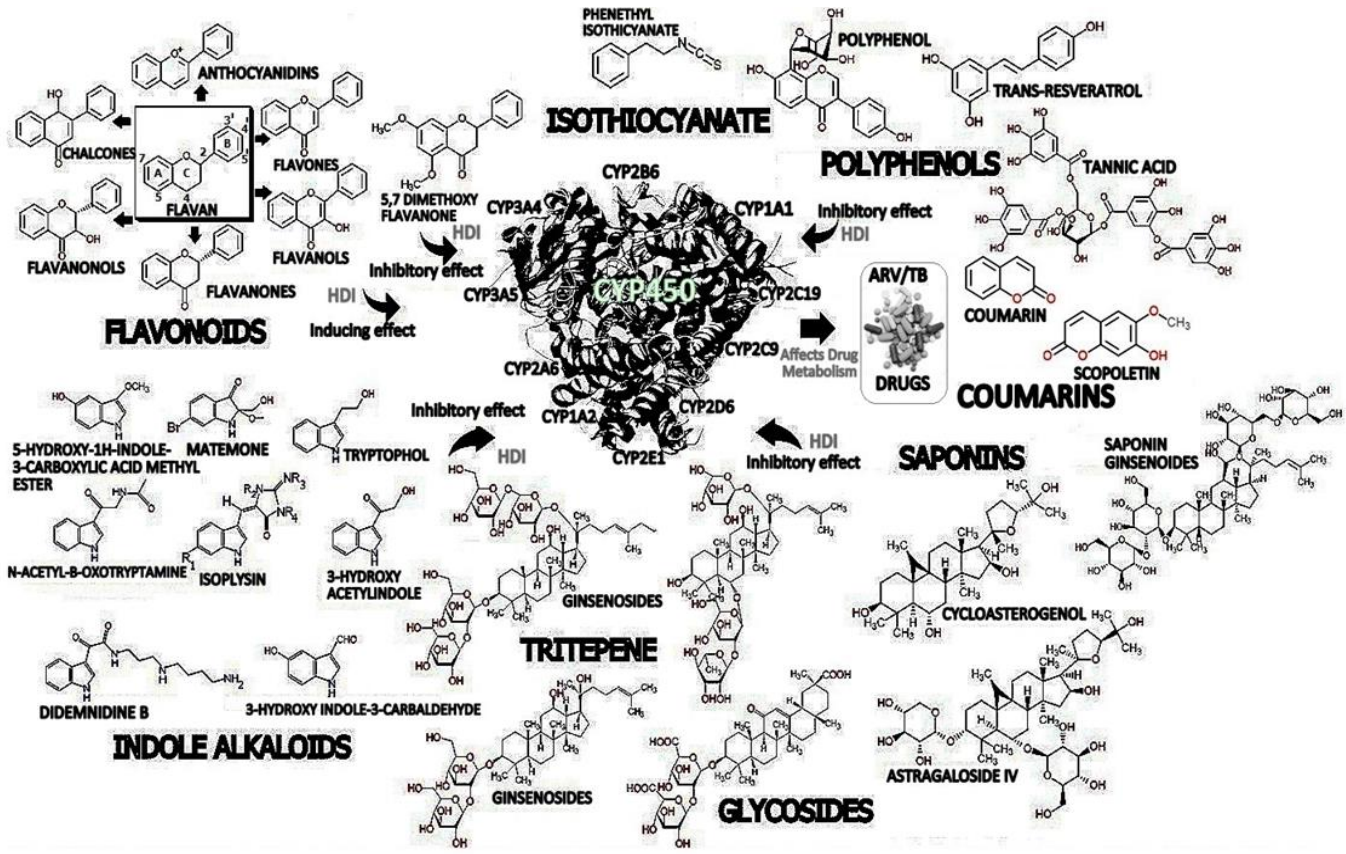


Fig. 3 Phyto-CYP Interaction assessment prediction model : This model outlines various class of phytocompounds and their molecules that have been previously reported to cause interactions with CYP-mediated metabolism in HLMs, rat and human subjects. The model links the potential of a herb to cause HDI to its phytochemical fingerprint.

As illustrated in Fig. 3, flavonoids have the potential to modulate the activity of cytochromes P450, mainly the CYP1A subfamily, significantly. The administration of rutin and quercetin enhances all measured specific activities of certain CYPs, but to a lesser extent compared to significantly high induction effect of isoquercitrin (Křížková et al., 2009). Many indole alkaloids have previously been reported as inhibitors of CYP2E1 and CYP2A6 (Wang et al., 2015). Coumarins are competitive inhibitors of CYP1B1 (Mammen et al., 2005), CYP1A1 and CYP1A2 (Cai et al., 1996). Phytophenols such as tannic acid and trans-resveratrol have been proven to have an inhibitory effect on the enzymatic marker of CYP2E1, while gallic acid also had a time-dependant inhibitory effect on CYP3A4 (Pu et al., 2015).

The inhibition and mechanism-based inactivation potential of phenethyl isothiocyanate (PEITC) on CYP activities have been investigated using microsomes expressing specific human CYP isoforms, extracted from baculovirus-infected insect cells (Nakajima et al., 2001; Thapliyal and Maru, 2001). Saponins such as deglycosylated ginsenosides can moderately inhibit CYP activities *in vitro* with IC_{50} values 10-50 μM (Yu et al., 2012), while cycloastragenol inhibits CYP3A4 activity in rat liver microsomes (Wei et al., 2014). 5,7-dimethoxyflavanone constituent present in a pacific beverage *kava* inhibits CYP1A2, CYP2C9, CYP2C19, CYP2D6

and CYP3A4 (Showman et al., 2015), and the triterpene astragaloside IV from *Astragalus* has an inhibitory effect on CYP2C9 and CYP3A4 (Shan et al., 2012).

1.6.1 Herb - drug interactions of African herbal medicines

Usage of herbal medicines, such as *Hypoxis hemerocallidea*, *Lessertia frutescens*, *Harpagophytum procumbensis*, *Spirostachys africana* and *Hoodia gordonii*, remains popular as an alternative or complementary form of treatment, especially in Africa (Pereira et al., 2010; Mills et al., 2005; Prevoo et al., 2004; van Wyk, 1997). *L.frutescens* and *H.hemerocallidea* were recommended by the South African Ministry of Health in the treatment of HIV/AIDS (Giraldo, 2002), including phase II trials conducted by the South African Herbal Science and Medicine Institute (SAHSMI) on *L.frutescens* (Aboyade et al., 2014).

The misconception that herbal remedies are completely safe due to their "natural" origins imperils human safety. In addition to potential inherent toxicity, different types of adverse interactions can occur with concomitant use of herbal medicines with conventional pharmaceuticals. Previous *in vitro* studies showed the inhibition of CYP3A4 and pregnane X receptor (PXR) by *L.frutescens* (Mills et al., 2005; Fasinu et al., 2013), CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9 and CYP3A4/5 by *H.hemerocallidea* (Fasinu et al., 2013; Mills et al., 2005), CYP1A2, CYP2D6 and P-gp transporters by *H.procumbensis* (Cordier and Steenkamp, 2004) and CYP3A4 by hoodigogenin A, extracted from *H.gordonii* (Madgula et al., 2010); while *S.africana* significantly induced CYP3A4 mRNA levels (van den Bout-van den Beukel et al., 2008).

Some of the CYPs being highly polymorphic pose a problem for pharmaceutical drug tailoring and design, to meet an individual's specific metabolic activity characteristics (The Royal Society, 2005). This is further complicated by the influence of herbal remedies. Unlike the more popular herbal medicines used in developed countries, very few data exists on the potential of African herbal medicines to cause HDI. Therefore, further research in the specific field of HDI for African herbal medicines is needed.

1.7 Herbal medicines selected for this research project

The herbs selected for this study *viz*., *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and *Ocimum basilicum* are commonly used in Africa by THPs as CAM for HIV/AIDS and TB. These herbs are available in the pharmacies

as health supplements, essential oils and tea extracts. *O.basilicum* is a common culinary spice, while *G.glabra* and *A.officinalis* extracts are used in confectionaries also. However further research is required on the effect of these herbs on the metabolism of HIV/TB medication.

1.7.1 *Withania somnifera*

Withania somnifera (L.) Dunal, known commonly as *ashwagandha* or the Indian *ginseng* (winter cherry *(Eng.)*; koorshout *(Afr.)*; ubuvuma *(Xhosa)*; ubuvimbha *(Zulu)*) is a popular herb in *Ayurvedic* medicine, as well as African herbal medicine (fig. 4).

Fig. 4 *Withania Somnifera* (from Materica Medica, 2013)

In southern Africa this plant grows at altitudes of 15 - 2300 m. The berries and leaves are applied externally to tumours, tubercular glands, carbuncles, and ulcers (Mirjalili et al., 2009).The roots are used to prepare the herbal remedy *ashwagandha*, which has been traditionally used to treat various symptoms and conditions, having antioxidant properties (Scartezzini and Speroni, 2000) and constituents such as steroidal lactones (Mirjalili et al., 2009). Studies have also been done on *ashwagandha* being used in the treatment of neurodegenerative disorders (ven Murthy et al., 2010) and even a stress reliever (Cooley et al., 2009). In India and many parts of Africa, the root extract of *Withania* is used as a cure for diseases such as candidiasis and HIV/AIDS (Masevhe et al., 2015). Recent studies have even shown *ashwagandha* to neutralize the toxic effects induced by β-amyloid-induced toxicity and HIV-1Ba-L (clade B) infection using a human neuronal SK-N-MC cell line (Kurapati et al., 2013). *In vitro* studies have also been conducted on the root extracts of *ashwagandha* to understand the anti-HIV activity of these extracts (Rege et al., 2010).

HDI data: A research study in 2013 showed the lack of any significant CYP3A interactions of Withaferin A, Withanolide A and Withanoside IV from *Withania* using rat and human liver

microsomes (Savai et al., 2013). Other studies showed no *in vitro* CYP1A, CYP3A4 and CYP2D6 inhibition of *Withania* in human and rat liver microsomes (Savai et al., 2014).

1.7.2 *Glycyrrhiza glabra*

Historical sources for the use of *Glycyrrhiza* species as medicine, for symptoms of viral respiratory tract infections and hepatitis, include ancient manuscripts from China, India and Greece (Fiore et al., 2008). Popularly known as 'liquorice', *Glycyrrhiza glabra* L. is common to northern parts of Africa (irksos *(African/ Arabic)*) (fig. 5).

Studies have been done on antiviral effects of the extracts. Reduction of membrane fluidity lead to inhibition of fusion of the viral membrane of HIV-1 with the cell, induction of interferon gamma in T-cells, inhibition of phosphorylating enzymes in vesicular stomatitis virus infection and reduction of viral latency (Fiore et al., 2008). The phytocompound *glycyrrhizin (or glycyrrhizic acid)*, found in liquorice, has been proposed as being useful for liver protection in tuberculosis therapy (Gupta et al., 2008). Glycyrrhizin has demonstrated hepatoprotective, antimicrobial, antiviral and anti-inflammatory properties, and increasing effects on blood-pressure *in vitro* and *in vivo*, as is supported by the finding that intravenous glycyrrhizin (based on the theory that oral delivery of the compound has limited entry into the circulation) slows the progression of viral and autoimmune hepatitis (Sato et al., 1996; van Rossum et al., 1998; Chien et al., 2011; Yasui et al., 2011). Liquorice extract has demonstrated activity in a clinical trial, when applied topically, against atopic dermatitis (Reuter et al., 2010). Additionally, liquorice has lipid-lowering effects in patients with hyperlipidaemia (Hasani-Ranjbar, 2010). Liquorice has demonstrated efficacy in treating inflammation-induced skin hyperpigmentation (Leyden et al., 2011). Liquorice extracts may be useful in preventing bacterial plaques in the oral cavities and neurodegenerative disorders (Messier et al., 2012). Anti-ulcer, laxative, anti-diabetic, anti-

Fig. 5 *Glycyrrhiza glabra* plant, fruits and roots (from Medicinal Herbs, 2010)

inflammatory, immunomodulatory, antitumor and expectorant properties of liquorice have also been noted (Mazumdar et al., 2012; Ming and Yin, 2013; Fiore et al., 2008).

HDI data: *G.glabra* and its principle bioactive compound, glycyrrhizin, showed a weak interaction potential with CYP3A4 and CYP2D6 isoenzymes (Pandit et al., 2011). In other studies, liquorice compounds especially isoprenylated flavonoids and aryl coumarins, affected the activities of CYP isozymes *in vitro*. Glabridin inhibited CYP3A4, CYP2B6, and CYP2C9 (Kent et al., 2002) and glycyrrhetinic acid inhibited CYP2C9, CYP2C19 and CYP3A4 (Zhao et al., 2012) while glycyrrhizin induced human CYP3A4 (Tu et al., 2010).

1.7.3 *Astragalus membranaceus*

Astragalus membranaceus (Fisch.) Bunge (Milk Vetch Root and Huang Qi *(Chinese)*) is one of the 50 fundamental herbs used in TCM (Duke and Ayensu, 1985) (fig. 6). It has been asserted as a medicinal tonic in improving the functioning of the adrenal glands, lungs and the GIT, increase metabolism and sweating, promote healing and immunity, and reduce fatigue (Balch, 2006).

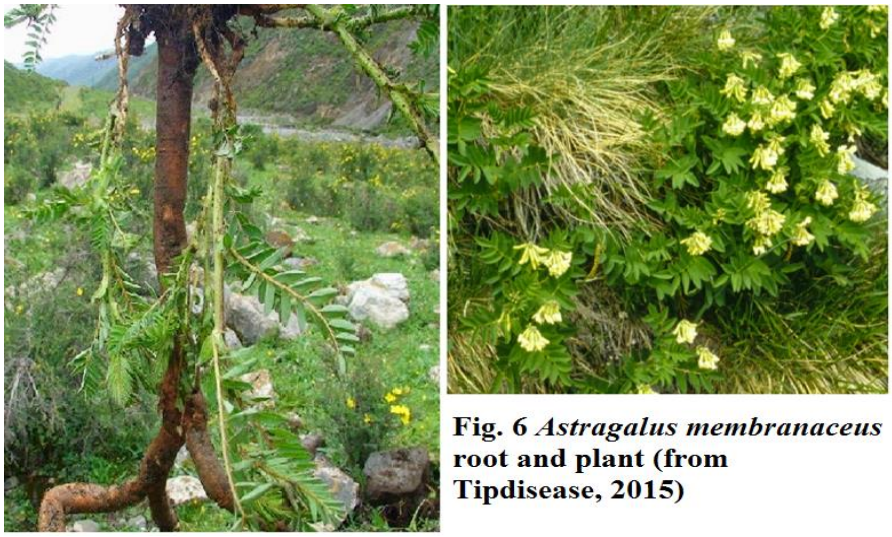


Fig. 6 *Astragalus membranaceus* root and plant (from Tipdisease, 2015)

Biotechnology firms are working on deriving a telomerase activator from *Astragalus* (de Jesus et al., 2011). The chemical constituent cycloastragenol (also called TAT2) is being studied to help combat HIV based on its telomerase activation properties, as well as infections associated with chronic diseases or aging (United Press Int., 2009). *Astragalus* roots are being sold as supplementary medicine for HIV and other chronic diseases, in pharmacies in South Africa. It has gained more popularity in Africa as a health supplement for supporting the immune system, preventing colds and upper respiratory infections, and is available in the form of capsules, tablets, tinctures and powder (NCCAM-NIH, 2010).

HDI data: In an *in vitro* study using HLMs, *A.membranaceus* inhibited CYP3A4-mediated metabolism of testosterone and increased the C_{max} and AUC of midazolam (Pao et al., 2012). The pure molecules astragaloside IV and cycloastragenol are inhibitors of CYP3A4, and CYP2C9 (Wei et al., 2014; Shan et al., 2012).

1.7.4 *Inula helenium*

Inula helenium L. *(Elecampane)* also called scabwort (horseheal, elf dock *(Eng.)*; rasan *(African/ Arabic)*) is a perennial composite plant common in many parts Africa, Great Britain, and can be found throughout central and Southern Europe and in Asia. The roots of this plant are used as folk medicine in many countries in Europe and Asia (fig. 7). The phytoconstituents include flavonoids, phenols, inulin (Afemei et al., 2012), alantolactones and helenin (Bruneton, 1995). It is used by herbalists for respiratory conditions; it loosens phlegm and is good in cases of colds, bronchitis and emphysema. It is also useful during the menopause to help reduce night sweats (Bartram, 1998). Some herbalists prescribe *Inula* as medicine for a wide range of other diseases including TB. A bioassay guided research-based analysis on the constituents of *Elecampane* such as eudesmanolides, alantolactone, isoalantolactone, and 11-alpha H,13-dihydroisoalantolactone, exhibited significant activity against *Mycobacterium tuberculosis* with minimum inhibitory concentrations of 32-128 µg/mL (Cantrell et al., 1999).

Fig. 7 *Inula helenium* plant and root (from Planetary Herbology, 2015)

HDI data: Alantolactone showed a potent inhibitory effect on CYP3A4 activity with IC_{50} values of 3.56 µM (HLMs) and 3.90 µM (recombinant CYP3A4) (Qin et al., 2015), while isoalantolactone showed potent inhibition of CYP2C19 (Kong et al., 2014).

1.7.5 *Althaea officinalis*

Althaea officinalis L., commonly known as ‘marshmallow’ (Baer-ul-Khtmk *(African/ Arabic);* Gulkhairo *(Pers.)*) is a perennial species indigenous to Africa, which is used as a medicinal plant and ornamental plant (fig. 8). A confection made from the root since ancient Egyptian

time evolved into today's marshmallow treat. The generic name, *Althaea*, is derived from the Greek word *'althein'* which means 'to cure', from its healing properties (Simonetti, 1990).

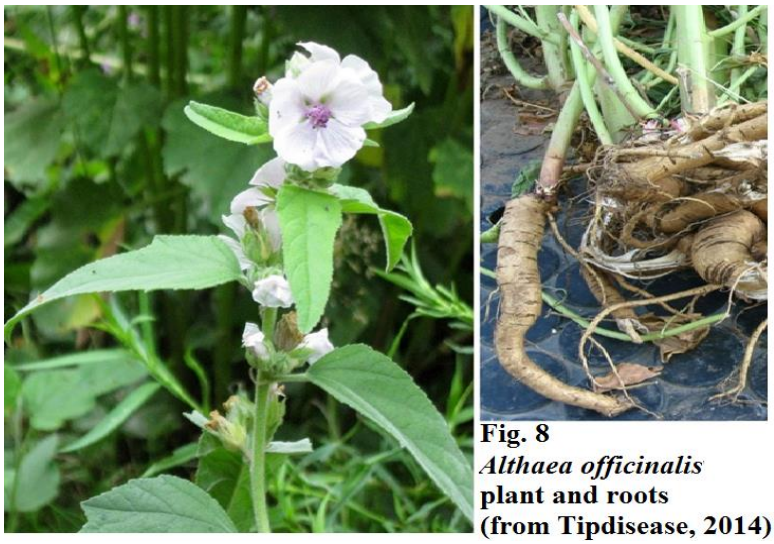


Fig. 8 *Althaea officinalis* plant and roots (from Tipdisease, 2014)

Marshmallow is traditionally used in the treatment of irritations in the mucous membranes, including use as a gargle tonic for mouth and throat ulcers, as well as gastric ulcers (Williamson and Wyandt, 1997). A study on rats concluded that an extract from the flowers has potential benefits for health conditions such as hyperlipidemia, gastric ulcers and platelet aggregation (Hage-Sleiman et al., 2011). The root has been used since the middle ages in the treatment of sore throat (Rachel, 2006). Extracts for marshmallow are claimed to cure tuberculosis, pertussis, pneumonia, dry cough, night sweats, five heart heats, small rapid pulse and other wide range of diseases and body conditions (Cravotto et al., 2010; Sutovska et al., 2007; Basch et al., 2003). A phytochemical study on the constituents of the plant led to the isolation of three phytocompounds, altheahexacosanyl lactone (n-hexacos-2-enyl-1,5-olide), 2β-hydroxycalamene (altheacalamene) and altheacoumarin (Rani et al., 2010).

HDI data: No relevant research data on potential interactions of *A.officinalis* with CYPs have been published.

1.7.6 *Ocimum basilicum*

Ocimum basilicum L. or sweet basil (Basilikum, Soet balsam *(Afr.)*; Mrihani *(Swahili)*) is a common culinary herb, native to India, China, Southeast Asia, New Guinea and many parts of Africa. It was originally domesticated in India, having been cultivated there for more than 5,000 years (Soule, 2011).

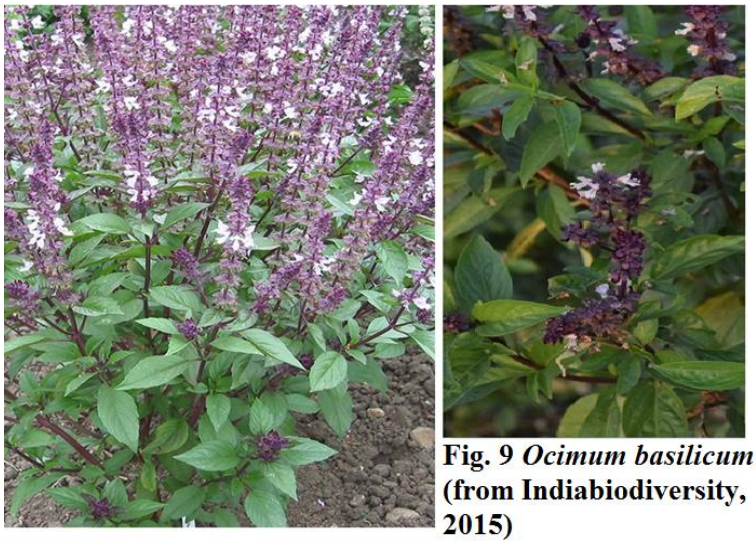


Fig. 9 *Ocimum basilicum* (from Indiabiodiversity, 2015)

Its leaves are mostly used in Italian and Southeast Asian cuisines (fig. 9). Basil is used for its medicinal properties in Ayurveda and Siddha medicine (a traditional *Tamil* system of medicine). The volatile oil of basil comprises of eugenol (Akgül, 1989) which is used in medicine as a local antiseptic and anaesthetic (Jadhav et al., 2004). The anti-TB activity of the crude methanolic extract from the aerial parts (leaves, fruits and flowers) of the plant has been reported (Siddiqui et al., 2012). *In vitro* studies have shown that compounds in basil oil have antioxidant, antiviral, and antimicrobial properties (Bozin et al., 2006; Chiang et al., 2005; de Almeida et al., 2007; Manosroi et al., 2006). *O.basilicum* is traditionally used for supplementary treatment of stress, asthma and diabetes in India (Duke, 2008).

HDI data: A methanol-dibutyl ether extract of basil showed inhibitory activity on CYP1A2 (Jeurissen et al., 2007). Another study showed that the methanolic extract of basil inhibits CYP2D6, CYP3A4, CYP3A5, and CYP3A7 (Nguyen et al., 2014).

1.8 Experimental *in vitro* assays to assess CYP enzyme activities

As the liver is the major drug-metabolizing organ, liver-derived systems are most effective and suitable for metabolic studies. Liver microsomes, recombinant enzymes, liver slices and primary hepatocytes are most recommended for *in vitro* metabolism data. In this study, HLM was used and analyses of the samples were carried out using an HPLC-PDA system.

1.8.1 CYP inhibition - liver microsome assays

During drug development and HDI studies, liver microsomes are often the first hepatic model used in metabolic studies. Microsomes are easily prepared from homogenized liver tissue, separated by differential centrifugation into microsomal, S9, cytosolic and mitochondrial fractions.

Selecting the most appropriate substrate specific to a particular CYP is critical for measuring its activity in *in vitro* studies (Frye, 2004). In recent years, the scientific literature has detailed information on the substrates metabolized by the major human CYPs (Walsky and Obach, 2004; Donato and Castell, 2003; Yao et al., 2007) and the U.S. Food and Drug Administration (FDA Clinical Pharmacology Guidelines, 2012) also outlines the *in vitro* and *in vivo* CYP specific substrates, inducers and inhibitors.

Classical inhibition assays involve the co-incubation of HLM and co-factors (glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, NADPH) at 37 ^{o}C with several concentrations of the tested plant extract and selective substrates (such as efavirenz for CYP2B6) in phosphate buffer for individual CYP enzymes (Masimirembwa et al., 2003; Bjornsson et al., 2003; Walsky and Obach, 2004). The assay is terminated after the incubation time, using chilled acetonitrile, centrifuged and the supernatant is analysed using HPLC-PDA (Polasek et al., 2006). Inhibition potency of the extracts can be ranked in order of IC_{50} or K_i. To a standardized comparison of the results obtained from different incubations, a typical CYP inhibitor (positive control, recommended as per FDA guidelines) must be included in each assay and compared to reference values. Before performing CYP inhibition assays, characterization and optimization of the kinetic parameters of the CYP substrate in the microsomal system is necessary (Bu et al., 2001).

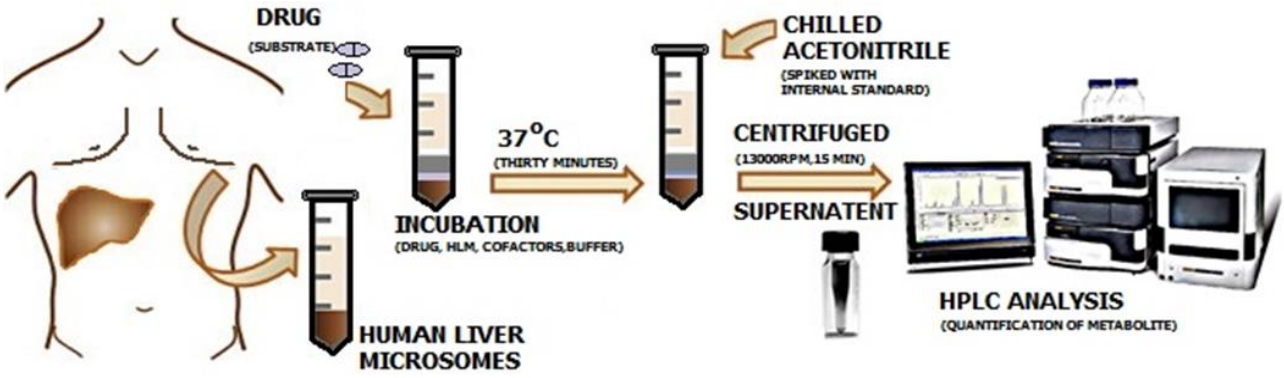


Fig. 10 Stepwise diagrammatic representation of HLM assays using HPLC analysis for inhibition studies.

The Cytochrome P450 TDI IC_{50} shift assay is used to identify both reversible and time-dependent inhibitors. The IC_{50} shift assay determines the IC_{50} value of a test compound based on three different experimental conditions; 0 min pre-incubation, 30 min pre-incubation with NADPH and no pre-incubation with NADPH. The TDI shift is a comparison of the IC_{50}s for the preincubation with NADPH to the one with no preincubation.

HLMs contain a wide variety of drug metabolizing enzymes and are commonly used to support *in vitro* ADME studies. The CYP activities in the HLM are determined using validated LC-MS/MS methods and provided in the datasheet. HLMs have long-term stability and can be cryopreserved. Relevant animal models for pharmacokinetic and toxicological studies can be identified by comparing the CYP metabolic profiles (liver microsomes) from different species with those of the human liver microsomes (Jia and Liu, 2009).

A major limitation of this *in vitro* model is that microsomes lack drug metabolizing cytosolic enzymes (glutathione S-transferases, sulfotransferases, xanthine oxidase, soluble epoxide hydrolases, alcohol dehydrogenase etc.).

1.8.1.1 **Analysis of HLM assay samples:**

Most *in vitro* assays involve HPLC separations, MS analysis or the use of radiometric probes for metabolite detection (Lahoz et al., 2006). Selective fluorescence activity assays are applied to biological models expressing single CYPs.

1.8.1.1.1 HPLC (photo diode array/ variable wavelength detection)

The HPLC-PDA platform uses full wavelength detection range from 190 nm to 800 nm and provides superior separation for analytes (drug and its metabolites, internal standard) which have UV-Vis absorption. The PDA passes a wide spectrum of light through the sample, after which it is separated into individual wavelengths and passed onto an array of photosensitive diodes. Each diode is specifically sensitive to one wavelength (Müllertz et al; 2016).

The VWD uses a deuterium or similar lamp which produces a broad spectrum of wavelengths separated by a diffraction grating. The instrument is adjusted so that only a particular wavelength of interest passes through the sample. The concentration of the sample is measured equivalent to the light that wasn't absorbed by the sample.

The HLM assay samples are run through specific columns and the separation of the drug, its metabolite and the internal standard is analysed using the peak retention times and areas for each, and quantification of the metabolite (ratio of the peak areas of the metabolite to the internal standard.

The HPLC method provides good sensitivity and stability for measuring light-absorbing compounds (~pg level), and is easy to operate. The advantage of using HPLC methodology for analysis of *in vitro* assay samples is that it is fast, sensitive and cheaper compared to many other techniques (Müllertz et al; 2016).

1.8.1.1.2 LC-MS analysis

HPLC coupled with electrospray ionization quadrupole time-of-flight (Q-TOF) tandem mass spectrometry has been demonstrated to be a powerful tool for analysis of biological samples

including the characterization of structural features and fragmentation behavior patterns (Li et al., 2011). LC–MS/MS (Triple quadrupole QqQ, Q-TOF), with its high sensitivity and specificity, has become a preferred analytical method for P450-involved HDI studies. The principle of an LC-MS is to convert the specific analyte to a gas-phase ion using electrical charge, which converts the resultant flux of the charged ions into a proportional electric current. This is converted into digital information by the system which displays it as a mass spectrum. The drug, its metabolite and the internal standard have specific masses which can be detected and quantified. Single or multiple reaction monitoring allows mass transitions that form the fingerprint of a specific analyte. There are several modes of MS detections including electrospray ionization (ESI) and atmospheric pressure chemical ionization ion trap mass spectrometry (APCI-IT-MS). Electrospray ionisation and tandem MS (ESCI-LC-MS) combines high speed switching between ESI and APCI within the ion source, enabling the use of MS methods with differing combinations and durations of both, within the same analysis. A QqQ MS has two quadrupole mass analysers in series with a non-mass analysing quadrupole in between which causes the fragmentation of the analyte (drug/ metabolite) through interaction with the collision gas (radiofrequency-only quadrupole). In Q-TOF MS, the analytes are converted to charged ions and accelerated by an electric field. The time taken by each ion to reach the detector is based on velocity which depends on its mass-to-charge ratio. The ion can be identified using this ratio and other known experimental parameters.

Advantages of LC-MS analyses are high sensitivity, broad quantitative range and the possibility to analyse fluorescent and non-fluorescent probes. Being more suitable for increased throughput drug screening (Turpeinen et al., 2006; Fung et al., 2003; Chu et al., 2000), the samples are analysed by using fast gradients and short columns, thereby reducing the HPLC run time significantly.

1.8.2 S9 fractions and hepatocytes

S9 fractions are obtained by the differential centrifugation of liver homogenates in isotonic potassium chloride. These subcellular fractions contain both phase I and II enzymes and therefore provide an ideal *in vitro* system for studying CYP activity as well as better representation of *in vivo* drug metabolism. S9 fractions provide a simple, convenient cost-effective and efficient alternative to other complex liver models and can be used to determine whether a drug undergoes oxidative metabolism and analyse its kinetic parameters, based on metabolite formation (Kawaguchi et al., 2018). However, the enzyme activity in S9 fractions is

substantially lower when compared to human liver microsomes (Brandon et al., 2003), which may result in undetected metabolite formation (Hakura et al., 1999).

In vitro hepatocyte models are reliable and readily available for inhibition studies. Cryopreserved hepatocytes are used to study inhibitory effect of herbal medications on cytochromes (Fasinu et al., 2013; Thelingwani et al., 2012).

1.8.3 Recombinant CYP systems

Human CYP enzymes heterologously expressed in different host systems show catalytic properties comparable to those of HLMs (Gonzalez and Korzekwa, 1995; Tang et al., 2005; Wang et al., 2000). Recombinant human CYPs are isolated in microsomal forms and are commercially available for CYP phenotyping, metabolic stability screening and inhibitory potential evaluation; moreover, they can be used for metabolism or toxicity studies including HDI assays (Naritomi et al., 2004; Bort et al., 2004).

A major advantage of recombinant CYPs in relation to HLM is the use of non-selective fluorimetric probes for high-throughput activity assays.

1.8.4 CYP induction

Induction of a drug metabolism enzyme refers to a process where the enzyme activity increases upon administration of a particular compound (inducer) through increased expression or synthesis, or by stabilization of the enzyme (Barry and Feely, 1990). Most drug transporters and oxidative and conjugative enzymes are inducible to a varying degree. However, the induction of CYP enzymes is of most concern, due to their prominent role in the drug metabolism. The induction of CYP enzymes are regulated by three principle nuclear receptors, namely, the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the aryl hydrocarbon receptor (AhR) (Lin, 2006).

The investigation of the induction of CYPs by herbal extracts is equally important as the inhibition assays. The key recommendations for CYP induction assays as per the US FDA, are to use human hepatocytes, mRNA level evaluation (recommended as endpoint) and initial study on CYP1A2, CYP2B6 and CYP3A4 (FDA Clinical Pharmacology Guidelines, 2012; Zhao, 2012). Induction of CYPs occurs when a drug or herbal extract triggers increased gene transcription thereby generating a higher amount of active protein compared to the normal

biological baseline. This may results in increased hepatic drug clearance. In the presence of an inducer of a particular CYP, the AUC of the co-administered drug metabolised by this CYP, is often reduced. St John's wort is an example of a CYP3A4 inducer which reduces the AUC of midazolam, a CYP3A4 substrate often used as marker drug for *in vivo* interaction studies, by 79% on co-administration (Mueller et al., 2006).

In this study induction of CYP was measured using mRNA expression in HepG2 (human liver carcinoma) cells (Fig. 11 *a, b, c*), through RT-PCR, followed by AGE.

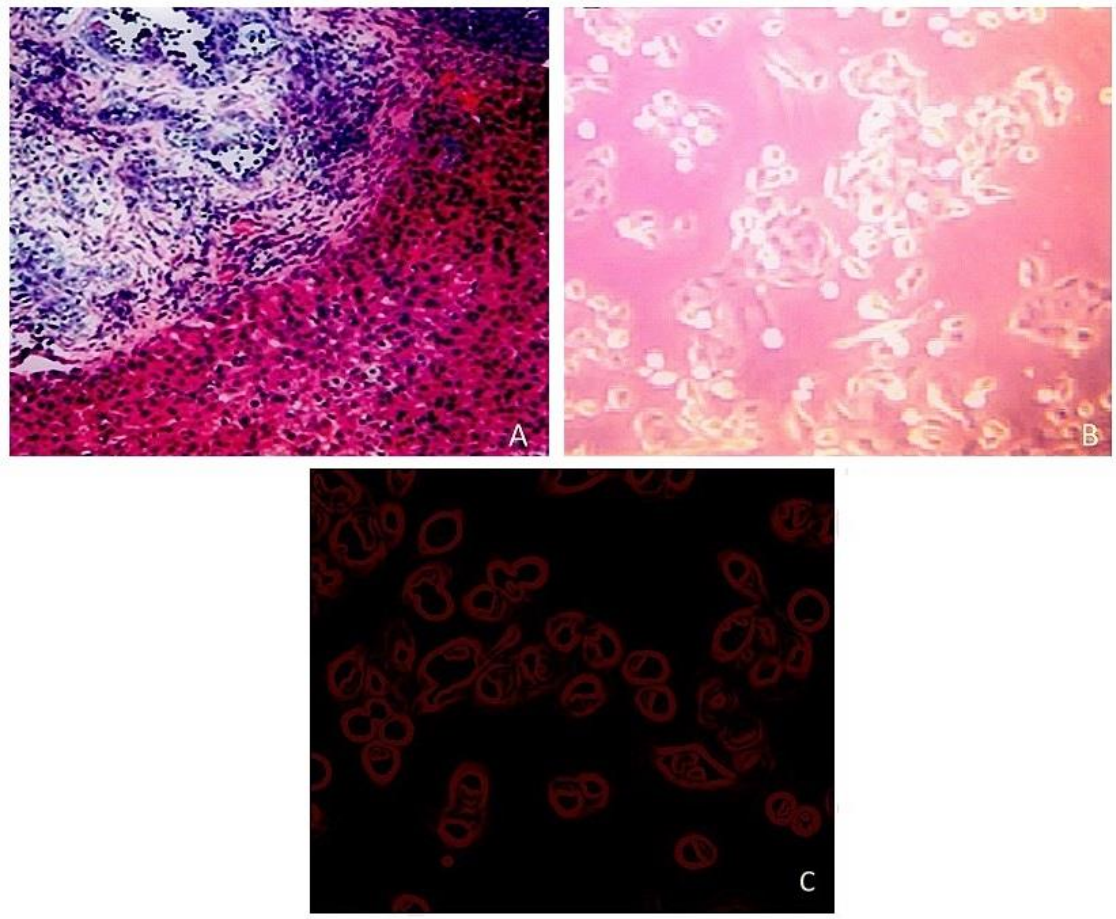


Fig. 11 A Clusters of HepG2 cells (stock culture), **B** & **C** Individual HepG2 cells in normal and neon view.

1.8.4.1 **mRNA quantification – Cell-based assays**

The mRNA quantification assays provide an invaluable tool in the assessment of induction in drug discovery programs of both *in vitro* and *in vivo* test samples, as well as in HDI studies. The quantification includes methods that use agarose gel electrophoresis (AGE) and radiolabeled probes as well as high-throughput methods based on polymerase chain reaction (PCR) using

complementary DNA (cDNA) technology or other advanced hybridization/amplification techniques, such as, branched DNA (bDNA) technology.

In the cDNA technology, the stock cells (such as HepG2) cells are cultured in 96-well microtiter plates and incubated, with the test sample (herbal extract) for 24 h at various concentrations. The cells are then treated with MTT dye and the absorbance is measured at 540 nm. The percentage of cell growth inhibition is calculated using the absorbance values obtained for each concentration of the extract and the extract concentration required to cause 50% cytotoxicity within the HepG2 cells (CTC_{50}) is generated from the dose-response curve.
The CTC_{50} concentration of each extract is used for subsequent 24 h incubations with the cells, and the total cellular RNA is isolated from these cells post incubation. cDNA is synthesized from the RNA using RT-PCR procedure and primers specifically designed for the CYPs. The amplified samples are then subjected to AGE and relative fold-expression levels are quantified (Reddy et al., 2015) (Fig. 11 *d*).

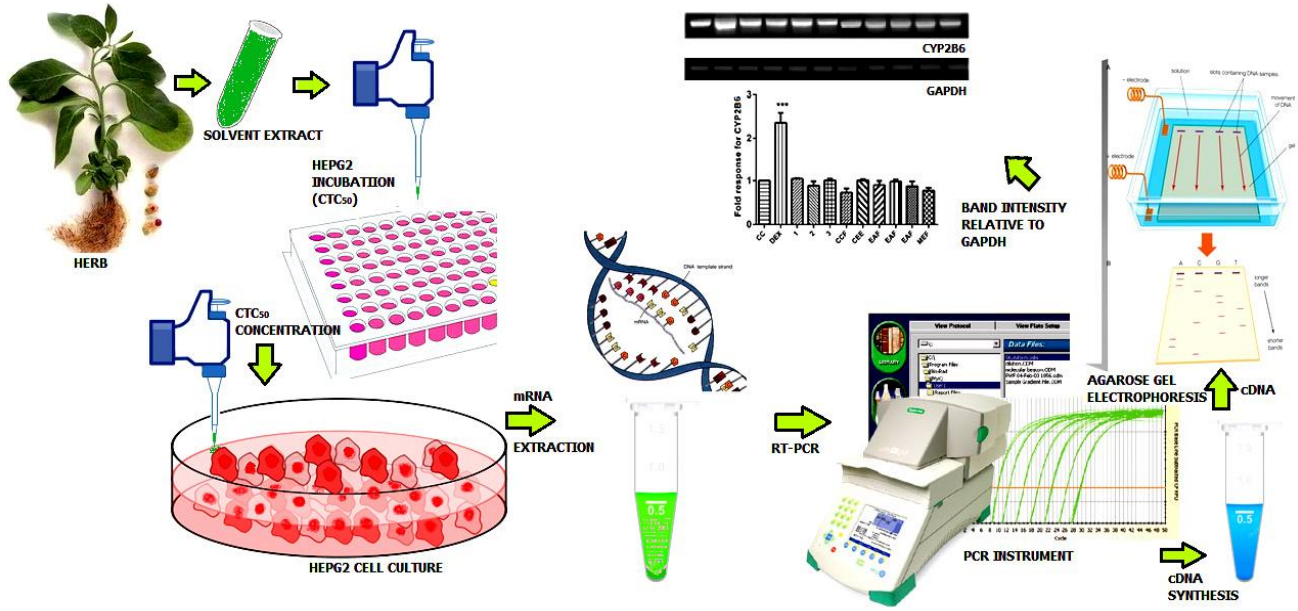


Fig. 11 D Stepwise diagrammatic repesentation of mRNA - Rt-PCR technique using HepG2 cells for induction studies using mRNA expression.

Analysis equipment availability and cost are often the deciding factor in the final choice of the analysis technique. The cDNA – RT-PCR method is highly efficient in assessing the fold-level induction of CYPs by herbs. However the cost involved in procuring CYP-specific primers can be high, and the method optimization can be time-consuming and tedious.

The bDNA technology is a hybridization-based method similar to an enzyme-linked immunosorbent (ELISA) assay (Urdea et al., 1991; Collins et al., 1997), where cell lysates are

applied to a plate coated with oligonucleotides with a specific sequence not related to the target mRNA, the capture probe. Two additional oligonucleotide probe sets are added, one that hybridizes with the capture probe and mRNA of interest (the capture extender probe), and the other that hybridizes with the mRNA of interest and has a sequence that can hybridize with a label probe (the bDNA). The label probe is coupled with an enzyme that allows for quantification of the mRNA, commonly alkaline phosphatase (AP).

This methodology is commonly used in clinical applications for the detection of HIV (Zhang and Surapaneni, 2012; Patel et al., 2009). Disadvantages of bDNA assays are the requirement of specialized reagents and technical know-how, and high cost involved.

1.8.4.2 **Reporter gene assays**

The reporter gene assay is an invaluable tool for analysing the molecular mechanisms of mRNA transcription for many genes. Additionally, it has become an effective methodology to help researchers define the mechanism by which enhancer elements in DNA interact with receptors that are responsible for the inducible nature of many drug metabolizing enzymes, such as CYP3A4, CYP2B6 and CYP1A2 (Garrison et al., 1996; Moore et al., 2000; Dickins, 2004). A reporter construct has two essential constituents within the regulatory region, *namely*, the enhancer element and the promoter element. These elements can vary widely. The promoter element can be derived from a general promoter, commonly thymidine kinase (TK) or cytomegalovirus (CMV) or even from a CYP of interest such as CYP3A4, CYP2C9, CYP1A2, CYP2B6, etc. The enhancer element, which is the element to which the receptor of interest will bind, is of critical importance in this methodology.

Well characterized PXR-based reporter gene assays have been developed. Reporter assays require the transfection of PXR, since PXR is not expressed in most if not all transformed cell lines. These assays are based on higher - density plate formats (384 -or 1536 - well) and provide a convenient high – throughput assay to screen molecules for PXR transactivation. A reasonable correlation of PXR activity in reporter gene assays with enzymatic activity and mRNA in primary human hepatocytes validates this assay for CYP induction studies (Luo et al., 2002). Several PXR ligand binding assays have been developed to help in the screening of various molecules in drug discovery programs (Jones et al., 2000; Moore et al., 2000; Zhu et al., 2004; Shukla et al., 2009).

1.8.4.3 **Protein quantification**

The most popular techniques for protein quantification include Western blot, ELISA, multiplexed immunoassay with beads, and quantitative proteomics.

The ELISA assay to measure protein levels, was first described in 1971 for immunoglobulin G (Engvall and Perlmann, 1971). The general procedure for this assay involves the following steps: The test sample (hepatocytes incubated with plant extract) containing an unknown amount of antigen (or target protein) is coated/fixed on to a microtiter plate through surface binding and left overnight at 4 ^{o}C. Then the blocking of the proteins is done using 3% BSA in PBS. The primary CYP antibody is then added, which binds to the antigen followed by the secondary detection antibody which is most often linked to a reporter enzyme such as AP or HRP. In the final step, a substrate is added (3,3',5,5'-Tetramethylbenzidine (TMB) or phosphatase), which is acted upon by the reporter enzyme to generate a quantifiable signal, for example, fluorescence or luminescence. The specific pure CYP isoenzymes are coated in various dilutions and blocked using the same procedure, and the optical density at a particular wavelength is measured, to prepare a calibration curve and the concentrations of the proteins/antigens in the unknown samples can be extrapolated using the standard curve.

The advantages of this method are its high sensitivity to provide high-throughput assays. Using a small amount of sample, it provides accurate protein quantification within a short turnaround time. Multiple samples can be analysed within a single plate, and comparative analysis of levels of induction by each sample in duplicates is achieved with better accuracy (less coefficient of variation (CV)). Being highly adaptable, this method has been applied to study drug - induced changes in expression levels of CYP isozymes, GSTs, and drug transporters (Nilsen et al., 1998; Kyokawa et al., 2001; Devi and Devaraj, 2006). However the cost involved in procuring CYP-specific antibodies is high. Also the CYPs induced by herbs are relative to the total content of the P450 enzyme system within the liver fraction or hepatocytes used in the assays; ELISA only quantifies the proteins from the assay.

Other methods used for protein quantification are as follows.

Mass spectrometry has been used for both absolute and relative quantitation of proteins and peptides, in enzyme induction research. Matrix-assisted laser desorption/ionization – Time of flight (MALDI – TOF) has been used to quantifyP450 isozymes in various rat and rabbit liver microsomes (Galeva et al., 2003) and highly homologous P450s such as CYP2B1 and CYP2B2

were easily differentiated. In this ionization technique, a laser energy absorbing matrix is used to create ions from large molecules with minimal fragmentation (Hillenkamp et al., 1991), and involves three steps: 1. the test sample with a suitable matrix material is applied on to a metal plate, 2. a pulsed laser is passed through the sample and the matrix material causing their ablation and desorption, and 3. finally, the resultant molecules are ionized and accelerated into a mass spectrometer for further analysis (Karas and Kruger, 2003).

In the LC-MS analysis the analyte and internal standard peptides co-elute, with the exception of mass difference whereby the concentration of the corresponding peptide analyte can be quantified relative to the internal standard. To aid in the design and optimization of proteomic experiments, several software packages are available to predict MRM transitions and peptides for a given protein based on unique peptide sequences (Mallick et al., 2007; Kohl et al., 2008). Recently, a detailed comparison between immunoassays and a nano-LC-MS assay was conducted for quantitation of CYP2D6.1 and CYP2D6.2 allelic isozymes (Yu et al., 2009).

Western blots are performed with chemiluminescence detection (Kricka, 2003). A general procedure of Western blot starts with gel electrophoresis of a protein sample followed by transfer to a nitrocellulose membrane. The membrane is then probed for the antigen of interest with a primary antibody, and subsequently, a secondary detection antibody is applied to recognize the primary antibody, using a reporter enzyme such as horseradish peroxidase (HRP). Some of the other common techniques include reporter gene assays, and hepatocyte-cell based assays.

1.8.4.4 **Hepatocyte cell - based assays**

Several *in vitro* hepatocyte models have been characterized for their ability to accurately assess new chemical entities for their induction liabilities. Primary human hepatocytes are the best characterized system to assess induction, and accepted by the regulatory agencies. Several reports have demonstrated the usefulness of such cells to assess the induction potential of these chemical entities (Hewitt, et al. 2007; LeCluyse et al., 2000). Cryopreserved human hepatocytes have been used for several induction studies including herbal medicines, to understand the effect on the cytochromes (Jackson et al., 2017; Cho et al., 2014; Klieber et al., 2010; Garcia et al., 2003).

Hepatocyte-like cell-based assays are also being used currently. The guidelines issued by the FDA (FDA Clinical Pharmacology Guidelines, 2012) allows for P450 induction data generated in immortalized cells. Fa2N–4 is a nontumorigenic immortalized cell line that has been evaluated extensively for its potential use as a reliable *in vitro* model system to assess CYP enzyme induction. The induction of various important genes such as CYP1A1/2, CYP3A4, CYP2C9, CYP2B6, UGT1A, and MDR1 as measured by mRNA expression levels (Nagarajappa et al., 2016) and CYP metabolism-activity has been demonstrated (Mills et al., 2004).

1.9 Phytochemical extraction, separation and analysis

Phytochemicals are non-nutritive, biologically active compounds found in plants. Some of these phytochemicals have potential disease preventing properties and used as medicine. The common phytochemical classes include flavonoids, catechins, anthocyanidins, polyphenols, coumarins, isothiocyanates, alkaloids, terpenoids, glycosides and saponins. One or more active molecules of these classes are present in the plants, some of which have the potential to interfere with the activity of the CYP system.

1.9.1 Extraction

The general techniques of plant extractions include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, and ultrasound extraction (sonication). For aromatic plants, hydrodistillation techniques (water and steam distillation), hydrolytic maceration followed by distillation are employed (ICS-UNIDO, 2008).

In this study four solvents were used for extraction namely, water, methanol, ethanol and ethyl acetate. Hot water decoctions and exhaustive extractions were used for preparing various extracts (Pandey and Tripathi, 2014).

The basic parameters influencing the quality of an extract are:

1. Plant part used as starting material (dried roots, leaves, bark and inflorescence).
2. Choice of solvent used for extraction (water, ethanol, methanol, ethyl acetate, chloroform, hexane, DMSO).
3. Extraction procedure (maceration, hot water decoction, infusion and exhaustive extraction).

1.9.2 Qualitative analysis

The qualitative biochemical analysis is of critical importance in HDI studies whereby the intensity of the various classes of phytocompounds present in the herb can actually be linked to a prediction model based on known research data (illustrated in fig. 3), as to indicate if the herb has the potential groups of phytocompounds (such as flavonoids, phenols, glycosides, terpenoids etc.) capable of causing clinically relevant interactions with the CYPs. This would be the precursor step to analysing the activity of the herbal extracts further using *in vitro* assays and detailed phytochemical fingerprinting using HPLC/MS where various reference standards can be used for the particular class of phytocompounds present in each herb (for *e.g.* rutin and quercetin for flavonoids and gallic acid for phenols), predominantly (Raaman, 2006).

Qualitative analysis of the extracted plant sample is performed by various biochemical tests (Raaman, 2006; Harborne, 1973) such as:

1. Formation of white precipitate in the presence of Mayer's reagent or brown coloration on addition of Wagner's reagent, indicating the presence of alkaloids.
2. Formation of green coloration in the presence of ferric chloride solution, indicating the presence of phenols.
3. Keller-Kiliani test, where addition of glacial acetic acid followed by ferric chloride and sulphuric acid to form prominent brown ring coloration indicates the presence of glycosides.
4. Formation of reddish-brown precipitate on the addition of vanillin reagent, indicating the presence flavonoids.
5. Leibermann-Burchard test, where the addition of chloroform followed by acetic anhydride and concentrated sulphuric acid results in the formation of dark bluish precipitate, indicating the presence of phytosteroids in the sample.
6. Salkowski Test, where the addition of chloroform followed by concentrated sulphuric acid, results in the formation of reddish-brown layer in the sample, indicating the presence of terpenoids.

1.9.3 Separation and purification

Methods of separation and purification of compound include:

1. Paper chromatography
2. Thin-layer chromatography
3. Gas-liquid chromatography
4. HPLC (PDA/VWD), HPLC-MS

5. Gel permeation chromatography

1.9.3.1 **LC-MS/PDA analysis**

The standard procedure of analysis using both HPLC and LC-MS analysis includes identification of a compound by comparison of specific data obtained from HPLC and MS analyses with those of reference compounds (Yang et al., 2010). LC-MS methodology, merging the separation of phyto-components with quantitative analysis or qualitative identification, provides an effective means of analysing complex samples, being the most popular and advanced chromatographic technique in analysing plant samples and herbal mixtures.

Full mass scan analysis of the sample in both positive and negative ionisation modes, allows the extraction all possible masses which can then be interpreted and linked to known compounds, using high quality mass spectral databases and online repositories. A full scan mode will monitor a range of masses know as mass to charge ratio (abbreviated *m/z*). A typical mass scan range can be set in the MS and will detect compound fragments within that range over a set time period. The various solvent extracts of each herb are analysed by LC-MS coupled with PDA as well as MS/MS fragmentation, and the resultant chromatograms are compared with the reference compounds for identification and relative quantification (Fig. 12).

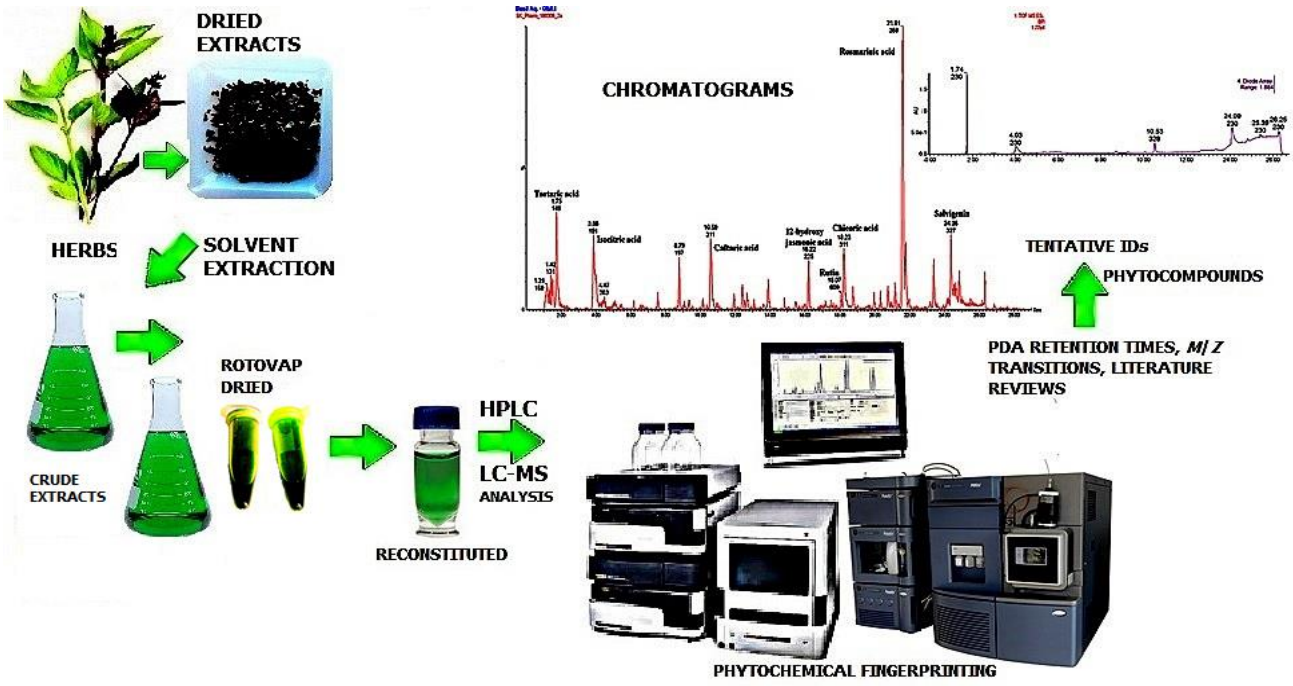


Fig. 12 Diagrammatic stepwise illustration of phytochemical analysis of various solvent extracts of herbs, using HPLC/LC-MS analysis - indentifying the possible molecules using matching retention times, m/z transitions and molecular formula.

The tentative identification of possible compounds depends on various factors such as match of the PDA retention times at a particular wavelength, *m/z* transitions matching known compounds and the derived molecular formula for the positive or the negative scans, based on literature reviews as well as online mass spectral databases (Stander et al., 2017; Bolleddula et al., 2012). Tentatively identified compounds are compounds that can be detected by the analytical testing methods developed using LC-MS, but the exact identity and concentration cannot be confirmed without further analytical investigation, such as isolation and purification. These compounds are detected and classified using LC-MS but the analysis is not target-specific (Murphy et al., 2003).

Phytochemical fingerprinting is a major step in understanding the natural composition of any herb. Fingerprint approach using LC-MS chromatography is one of the most effective tools in assessing the quality of herbal medicines. In many industries, such techniques are mandatory for quality assurance of various synthetized chemicals.

1.9.3.2 **Limitations**

Since the fragment information of MS is easily affected by ionization modes and HPLC conditions, it is necessary to establish universal database with the help of the reference substances development, for the HPLC-MS like the GC-MS. Another limitation of HPLC/MS is that the peak capacity of an HPLC column is limited. In LC-MS analysis, chemical identification and structural elucidation of a particular compound based on mass spectral database can be tedious since various compounds have the same *m/z* ratio. Also in LC-MS analysis, some of the extracts might have peaks within the same transitions of the reference standards, which could indicate the possibility of another compound (plant metabolite) matching the same transition such as an isomeric form or an isobar (Bjarnholt et al., 2014).

1.10 Motivation and rationale of this study

The South African healthcare system is under considerable pressure because of the high prevalence of tuberculosis and HIV/AIDS, along with non-communicable diseases such as cardiovascular diseases, diabetes, cancers, chronic respiratory diseases and mental illness (Wyk et al., 2013). These diseases affect the overall quality of life of an individual and have resulted in increased healthcare expenses. The challenges associated with such health conditions and the sociocultural outlook and family history of many patients especially in Africa have increased the use of THMs for many diseases (Hughes et al., 2015). Herbal medicines are easily available

in the form of supplement tablets, teas, extracts, and essential oils, in most of pharmacies, health stores, or local market.

Although often herbal medicines are presumed safe by consumers, considering they are natural, there are serious risks associated with their inappropriate use. Therefore, there is a need to develop standards to assure the safety of herbal medicines, especially in Africa (Kuete, 2017). One of the concerns is the possibility of HDIs when herbal medicines are co-administered with conventional drugs, as discussed above.

The objective of the present research study is to understand and critically evaluate how the following herbs: *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and *Ocimum basilicum*, can interact with drug pharmacokinetics, especially with the CYP enzymes responsible for most of drug metabolism. These herbs have been used as medicines by THPs for centuries in Africa, for various medical conditions such as HIV and TB. No adequate research has been performed on the appropriate dosage regimens for these herbs and their potential to interact with the CYPs involved in drug metabolism. The THPs involved in this study, had shown their interest in understanding the possibilities of HDI, whereby they could also ensure safer use of herbs and make their customers aware of the potential interactions.

One of the greatest challenges faced by many countries in Africa, especially South Africa is the control of concomitant HIV and tuberculosis epidemics. Drug-resistant tuberculosis (DR-TB) is a major threat to the efforts to end TB in South Africa (Cox et al., 2017) and the mortality rate is high for this epidemic. The increasing DR-TB cases, HIV-TB co-infection and TB mortality constitutes a situation crisis in Africa which needs urgent attention. The two drugs used in this research, efavirenz and rifampicin are part of first line regimens for treatment of HIV and TB in South Africa. The pharmacokinetic profile of these drugs can be affected if the enzymes that metabolize them undergo inhibition or induction. This can ultimately affect the effective dose regimens of the drugs or even have toxic effects.

CYP2B6 is predominantly involved in the metabolism of efavirenz, whereas esterases are involved in the metabolism of rifampicin. However, not much research has been done on the effect of HDI on the metabolism pathway of rifampicin to 25-O-desacetyl rifampicin. This study focuses on this pathway, to understand the inhibition potential of the selected herbs,

which are typical and relevant examples of herbal medicines used by THPs for the treatment of TB and HIV. Introducing rifampicin as a substrate with HLMs, to assess its metabolism profile and analyse if these herbs have the potential to induce or inhibit the formation of 25-O-desacetyl rifampicin is critical, because if the pharmacokinetics of this metabolism pathway is affected, it may lead to incomplete metabolism of rifampicin, which may result in toxicity, including mortality (Sridhar et al., 2012; Cheng et al., 1988).

The research presented in this thesis can be divided into four parts: (1) biochemical qualitative phytoprofiling of the various groups of phytocompounds present in the extracts, (2) inhibition assays using HLM to assess if these herbs can inhibit CYP2B6 and the metabolism pathway of rifampicin to 25-O-desacetyl rifampicin, (3) induction assays using HepG2 cells to study the possibility of induction of mRNA expression for CYP2B6 and CYP3A4 by the herbs, and (4) LC-MS/PDA phytochemical analyses of the chosen herbal extracts to identify and relatively quantify the major phytoconstituents present in each extract and predict the compounds capable of causing clinically relevant HDI.

1.11 Potential benefits of this study

Metabolism studies are required to understand the interactions between herbal medicines and synthetic drugs, before conclusions are derived on the appropriate use of such herbal medicines, especially when consumed with conventional drugs. This research is beneficial in providing new information on the possibility of HDI of the traditional African herbal medicines *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and *Ocimum basilicum.*

It forms a critical step in the evaluation of these herbal medicines and their constituents for inhibitory or inducing effect on drug metabolising enzymes, to develop appropriate, scientifically based product information of such supplements. The results of this research will important for planning and designing future clinical pharmacokinetic interaction studies. Together, the new information gained by these research investigations will be important in formulation of health and safety policy recommendations for the use of herbal supplements, including the risk of HDI, and to provide advice to health care practitioners and patients on the risk of HDI when co-administering conventional medicines together with herbal medicines.

1.12 Hypothesis

The hypothesis of this study was that *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and *Ocimum basilicum*, which are consumed as THM for many diseases including HIV and TB, will interact with the CYP2B6–mediated metabolism of conventional drugs such as efavirenz and/or with the B-esterases-mediated rifampicin metabolism pathway, in the human liver.

1.13 Research: Aim and objective

The primary aim of this research was to analyse selected African herb varieties used as traditional medicines for various diseases including HIV/AIDS and TB, namely *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and *Ocimum basilicum*, and investigate their potential interactions on CYP2B6 mediated metabolism of efavirenz (to 8-Hydroxy efavirenz), esterase-mediated rifampicin (to 25-O-desacetyl rifampicin) metabolism pathway, and inducing effect on CYP2B6/CYP3A4 mRNA expression. This was achieved through *in vitro* assays using HLM and HepG2 cells. The active extracts were subjected to detailed phytochemical analysis using LC-MS/PDA, to link the potential phytoconstituents to the activity on CYPs. Statistical analysis of the IC_{50} and TDI fold-shift, as well as fold-response of each extract on CYP2B6/CYP3A4 mRNA expression in HepG2 cells, was performed to compare the possible inhibition and induction effects of the investigated herbal extracts.

1.14 Ethical consideration

This study was approved by the University of Stellenbosch Health Research Ethics Committee (Reference number X15/04/002, Appendix 2).

Chapter 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Pure 8-Hydroxy efavirenz (E8H), 25-O-desacetyl rifampicin (25ODESRIF) and neostigmine methyl sulphate (NEO) were obtained from Clearsynth Labs Ltd. (Mumbai, India) while efavirenz (EFV), rifampicin (RIF), ticlopidine (TICL) and nelfinavir mesylate hydrate (NELF) were obtained from Sigma-Aldrich (Steinheim, Germany).

Purified water (Adrona B30 purification systems, Adrona SIA, Latvia), HPLC-grade methanol (Sigma-Aldrich, Germany), ethanol (Merck KGaA, Darmstadt, Germany) and ethyl acetate (BDH Chemicals, England) were used for the extractions.

The following reagents were used to conduct the biochemical tests for detecting classes of phytochemicals:

- Vanillin reagent: 1% Vanillin in 70% concentrated sulphuric acid (BDH chemicals, England) – to test for flavonoids.
- Wagner's reagent: 2 g of iodine (BDH chemicals, England) and 6 g of potassium iodide (Merck, Germany) in 100 mL of water – to test for alkaloids.
- Dilute Ammonia Solution (Hopkin and Williams, England) – to test for alkaloids.
- 0.1% ferric chloride solution, neutral ferric chloride solution (Sigma-Aldrich, Germany) – to test for tannins and phenols.
- 10% sodium hydroxide solution (BDH chemicals, England) – to test for coumarins.
- Magnesium solution (Hopkin and Williams, England) – to test for tannins.
- Glacial acetic acid, concentrated sulphuric acid (BDH chemicals, England), ferric chloride (Sigma-Aldrich, Germany) – to test for glycosides.
- Chloroform, acetic anhydride (BDH chemicals, England) – to test for steroids.

For the HPLC/MS analysis, LC grade acetonitrile and methanol was purchased from Sigma-Aldrich (Steinheim, Germany) and Merck KGaA (Darmstadt, Germany), and purified filtered water was prepared using Millipore purification systems (Billerica, MA, USA). Leucine

enkaphalin (lock mass reference standard) for LC-MS analysis was purchased from Sigma-Aldrich (Steinheim, Germany).

2.1.2 **Human liver microsomes and HepG2 cell lines**

The HLM screening was performed using pooled human liver microsomes (mixed gender - H0630) from Xenotech LLC (Kansas, USA). HepG2 (human hepatocellular carcinoma cells) cell line was procured from National Centre for Cell Sciences (NCCS), Cell Repository, Pune, India.

2.1.3 **Reagents and apparatus for inhibition/ induction assays**

For the inhibition assays, glucose-6-phosphate sodium salt, glucose-6-phosphate dehydrogenase, magnesium chloride, phosphate buffer solution 1 M, and β-nicotinamide adenine dinucleotide phosphate hydrate (NADPH) were purchased from Sigma-Aldrich (Steinheim, Germany).

For the induction assays, microtiter 96–well U–bottom plates from Tarsons Products Pvt. Ltd., Kolkata, India were used for the induction assays. 25 cm^2 cell culture flasks were purchased from Corning® Inc. (New York, US).

MTT (thiazolyl blue tetrazolium bromide), phosphate buffered saline pH 7.4 (PBS), nutrient mixture F-12 Ham, EDTA, Dulbecco's modified Eagle's medium – high glucose (DMEM), trypsin, and the antibiotics for cell culture were purchased from HiMedia Laboratories (Mumbai, India). Dimethyl sulfoxide (DMSO) was purchased from Finar Ltd. (Gujarat, India). Gibco™ Fetal bovine serum (FBS) was purchased from Thermofisher Scientific (MA, USA). Tri-Xtract™ for the isolation of RNA was purchased from G-Biosciences Ltd. (MO, US).

Esco class II biosafety cabinet (Esco Technologies, PA, USA) was used for cell culturing. CO_2 Incubator manufactured by Nuaire (MN, USA) was used for all incubations. AE-series inverted microscope (Motic Asia, Hong Kong) was used for tissue culture inspection. BioTek Epoch automated microplate reader with Gen5 2005 software v1.10.8 (BioTek Instruments, Inc. USA) was used for plate incubations and readings. Polymerase chain reactions were done using the MJ mini thermocycler (Bio Rad, Hercules, CA, USA).

2.1.4 **Data analysis**

For the statistical data plots, including the non-linear regression graph plots for determining the IC_{50}, GraphPad Software Inc. (San Diego, CA; *www.graphpad.com*) Prism version 5.00 for Windows, was used.

Digital imaging and relative sample expression levels for gel electrophoresis was done using inGenius - gel documentation system comprising of GeneTools analysis software (Syngene, MD, USA).

2.1.5 **Herbs**

The dried roots of *Withania somnifera*, *Glycyrrhiza glabra*, *Astragalus membranaceus*, *Inula helenium*, *Althaea officinalis* and leaves, seeds and inflorescence of *Ocimum basilicum* were obtained in packed form, from Pharma Germania, Benoni, Gauteng; the contents being pure, and certified (Table 2). A voucher specimen could not be submitted, because the dried herb parts were in semi-powdered form.

Table [2] Herbs, CoA# and Code names

Plant Species	Part	Certificate of Analysis (CoA #)	Code for Sample Preparation/ Reference			
			Aqueous	Methanol	Ethanol	Ethyl acetate
Withania somnifera (Ws)	Dried roots	137202	WsA1	WsM2	WsE3	WsEtA4
Glycyrrhiza glabra (Gg)	Dried roots	4243500	GgA1	GgM2	GgE3	GgEtA4
Astragalus membranaceus (Am)	Dried roots	B311400/FP136095	AmA1	AmM2	AmE3	AmEtA4
Inula helenium (Ih)	Dried roots	462563	IhA1	IhM2	IhE3	IhEtA4
Althaea officinalis (Ao)	Dried roots	471255	AoA1	AoM2	AoE3	AoEtA4
Ocimum basilicum (Ob)	Dried leaves and inflorescence	PFI-2645	ObA1	ObM2	ObE3	ObEtA4

***Note*:** The code name for each extract was derived using the initials of the scientific name, the type of solvent in serial order (1^{st}: aqueous – A1, 2^{nd}: methanol – M2, 3^{rd}: ethanol – E3, 4^{th}: ethyl acetate – EtA4).

2.2 Methods

The methods are divided into four phases of study:

1. Biochemical analysis of each extract using various chemical reagents that are reactive-specific for certain groups of phytocompounds (based on precipitate formation and reaction color intensity).

2. *In vitro* assays with HLM and the analysis of samples using HPLC.
3. Induction assays for mRNA expression of CYP2B6 and CYP3A4 in HepG2 cells.
4. Phytochemical fingerprinting of the active herbal extracts using LC-MS/PDA analysis, which includes tentative identification of major compounds based on the peak intensity, mass transitions and UV-maxima.

PHASE – I: EXTRACTION AND BIOCHEMICAL PHYOPROFILING

2.2.1 Extraction of herbal supplements for bioassays

2.2.1.1 Exhaustive extraction

Water decoction extract (A1): The dried roots of *W.somnifera*, *G.glabra*, *A.membranaceus*, *I.helenium*, *A.officinalis* and dried leaves of *O.basilicum* were powdered, weighed (4 g) and extracted exhaustively with water (Adrona B30, upto 500 mL for 9 days) using mechanical agitation. The extracts obtained were filtered and concentrated/ evaporated at 50 °C using a centrifugal vacuum concentrator (miVac, England) to complete dryness and marked as WsA1, GgA1, AmA1, IhA1, AoA1 and ObA1, and stored in a desiccator at 2-4 °C.

Methanol extract (M2): The dried roots of *W.somnifera*, *G.glabra*, *A.membranaceus*, *I.helenium*, *A.officinalis* and dried leaves of *O.basilicum* were powdered, weighed (4 g) and extracted exhaustively with methanol (HPLC grade, upto 500 mL for 9 days) using mechanical agitation. The extracts obtained were filtered and concentrated/ evaporated at 45 °C using a centrifugal vacuum concentrator (miVac, England) to complete dryness and marked as WsM2, GgM2, AmM2, IhM2, AoM2 and ObM2, and stored in a desiccator at 2-4 °C.

Ethanol extract (E3): The dried roots of *W.somnifera*, *G.glabra*, *A.membranaceus*, *I.helenium*, *A.officinalis* and dried leaves of *O.basilicum* were powdered, weighed (4 g) and extracted exhaustively with ethanol (HPLC grade, upto 500 mL for 9 days) using mechanical agitation. The extracts obtained were filtered and concentrated/ evaporated at 45 °C using a centrifugal vacuum concentrator (miVac, England) to complete dryness and marked as WsE3, GgE3, AmE3, IhE3, AoE3 and ObE3, and stored in a desiccator at 2-4 °C.

Ethyl acetate extract (EtA4): The dried roots of *W.somnifera*, *G.glabra*, *A.membranaceus*, *I.helenium*, *A.officinalis* and dried leaves of *O.basilicum* were powdered, weighed (4 g) and extracted exhaustively with ethyl acetate (HPLC grade, upto 500 mL for 9 days) using mechanical agitation. The extracts obtained were filtered and concentrated/ evaporated at 45 °C

using a centrifugal vacuum concentrator (miVac, England) to complete dryness and marked as WsEtA4, GgEtA4, AmEtA4, IhEtA4, AoEtA4 and ObEtA4, and stored in a desiccator at 2-4 °C.

Percentage of yield was calculated as per equation (2.2.1):

Extract %yield = $(W_1/W_2)\times100$ *Eq. (2.2.1)*

Where, W_1 is net weight of residue extract in grams after extraction and W_2 is total weight of dried herb in grams taken for extraction.

2.2.2 **Biochemical phyto-profiling**

A series of standard biochemical tests were performed to assess and evaluate the phytochemistry of the herbs, and rank them based on the intensity of the compounds present in each extract, as outlined below (Raaman, 2006; Harborne, 1973). The analysis was mainly done to assess the presence of nine specific classes of phytocompounds, *viz*., alkaloids, saponins, phenols, tannins, glycosides, terpenoids, flavonoids, steroids and coumarins. These compounds had previously been reported of causing HDI for many popular herbs. The objective of this analysis was to evaluate the content of each extract based on the precipitate formation, color and intensity, for each extract depending on the biochemical reactions (as below) for identifying the specific class of phytocompound and rank each herb for its potential to interfere with CYP activity.

For all the biochemical analyses, the test solution was prepared for each extract by dissolving it in its respective solvent, at a final concentration of 1 mg/mL.

2.2.2.1 Test for alkaloids

a. Wagner's test

To 150 µL of test solution, equal amount of Wagner's reagent was added. Formation of reddish-brown coloration indicated the presence of alkaloids.

b. Harborne test

120 µL of dilute ammonia solution was added to 150 µL of test solution of each plant extract followed by addition of concentrated sulphuric acid (Harborne, 1973). Formation of yellow coloration indicated the presence of alkaloids.

2.2.2.2 Test for saponins

About 150 µL of the test solution was mixed with 120 µL of distilled water and shaken vigorously for a stable persistent froth, which indicated the presence of saponins.

2.2.2.3 Test for glycosides (Keller-Kiliani test)

To 150 µL of the test solution, 100 µL of glacial acetic acid was added. To the resultant, a pinch of ferric chloride was added. Finally, 50 µL of sulphuric acid was added to the resultant solution. Formation of a prominent brown ring indicated the presence of glycosides.

2.2.2.4 Test for tannins

To 150 µL of test solution, 100 µL of 0.1% ferric chloride was added and observed for formation of blue-black precipitate, which indicated the presence of tannins.

2.2.2.5 Test for flavonoids

To 150 µL of the test solution, equal amount of Vanillin reagent was added. Formation of reddish-brown colour precipitate indicated the presence of flavonoids.

2.2.2.6 Test for phenols

To 150 µL of the test solution, 100 µL of neutral ferric chloride solution was added. Formation of greenish colour indicated the presence of phenols.

2.2.2.7 Test for steroids (Leibermann-Burchard test)

To 150 µL of the test solution, 100 µL of chloroform was added. Then 3-4 drops of acetic anhydride and 3 drops of concentrated sulphuric acid were added. Dark bluish precipitate was formed if phytosteroids were present.

2.2.2.8 Test for terpenoids (Salkowski test)

200 µL of the plant extract solution was mixed with 75 µL of chloroform, and 125 µL of concentrated sulphuric acid was carefully added from the sides of the test-tube. The gradual formation of a reddish-brown layer indicated the presence of terpenoids in the plant extract.

2.2.2.9 Test for coumarins

To 150 µL plant sample extract solution, equal quantity of 10% sodium hydroxide solution was added and heated at 100 ^{o}C for 5 min. Formation of yellow color indicated the presence of coumarins in the plant extract.

PHASE – II: CYP INHIBTION STUDY

2.2.3 **HLM assay validation**

The HLM assay was validated, by incubating EFV and RIF as substrates and NEO as internal standard. Briefly, a standard 200 µL incubation mixture containing liver microsomes (ranging from 0.25 – 1.00 mg/mL protein concentration), EFV/ RIF (100 μM) in 0.2 M phosphate buffer pH 7.4 at 37 °C was incubated in duplicate. The reactions were initiated with NADPH (final concentration 1.3 mM) along with the co-factors $MgCl_2$ (final concentration 3.3 mM), glucose-6-phosphate (final concentration 1.3 mM) and glucose-6-phosphate dehydrogenase (1 U/mL), and then terminated with 200 µL of chilled acetonitrile spiked with the internal standard NEO (20 μM). The samples were centrifuged using 0.22 µm polypropylene centrifuge tube filters (Spine-X® Costar, USA) at 13000 rpm for 5 min and 15 µL of the supernatants were subjected to HPLC analysis.

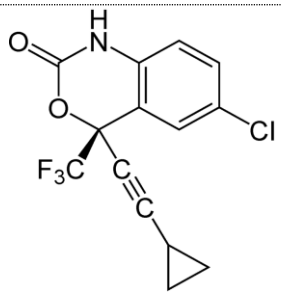
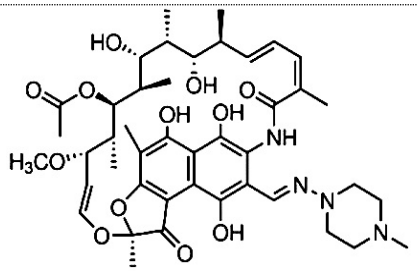
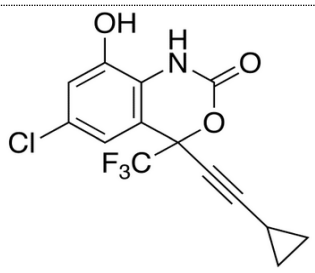
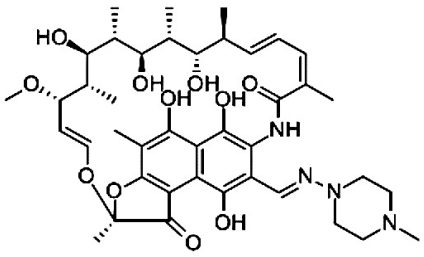
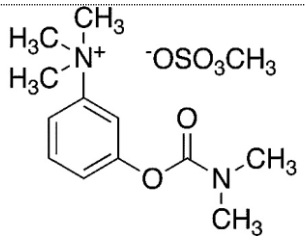
1. **HPLC–EFV method**: The HPLC gradient method was slightly modified from a previous study on CYP1A interaction (Varghese et al., 2014). The HLM-EFV assay incubates for determination of CYP2B6 activity, were run on a C-18 Phenomenex-Evo column (150 x 2.6 mm, 3.5 µm) by gradient elution using mobile phase comprising of water (A): acetonitrile (B) at a flow rate of 0.7 mL min^{-1} for clear separation EFV, its major metabolite E8H and the internal standard. The detection wavelength was 245 nm.

2.**HPLC–RIF method**: For determination of CYP3A4 activity, the RIF incubates were run on a C-18 Phenomenex Luna column (150 x 4.6 mm, 5 µm) by gradient elution using mobile phase comprising of water (A): methanol (B) at a flow rate of 0.8 mL min^{-1} for separating rifampicin, its metabolite 25ODESRIF and the internal standard NEO. The detection wavelength was 254 nm. The gradient solvents program was set as (*TT*min/% solution B) and 0/10, 5/80, 10/95, 9/80, and 11.5/10 for both methods.

Both the HPLC methods for EFV and RIF were validated for consistency, linearity and precision. A linear response was obtained in the concentration range 0 – 200 μM for both EFV

and RIF, and their metabolites (R^2 = 0.9930 and 0.9950 respectively). This concentration range was selected based on the literature search of the maximum concentration used in *in vitro* incubation assays for both drugs (100-150 μM). LLOD and LLOQ were determined for EFV (7.57 μM, 22.95 μM), E8H (7.99 μM, 24.24 μM), RIF (5.86 μM, 17.75 μM) and 25ODESRIF (7.78 μM, 23.57 μM), based on the International Council for Harmonisation (ICH) guidelines. Based on the rate of metabolite formation the optimum protein concentration was determined (0.5 mg/mL for EFV and 0.25 mg/mL for RIF), and the same assays were repeated for various incubation time slots – 15, 30, 45 and 60 min with fixed protein concentration, in duplicate. Based on the rate of metabolite formation, the optimum time of incubation was selected. The optimum protein concentration and time of incubation for both EFV and RIF assays, were used further to determine the kinetics of both drugs (Table 3).

Table [3] HLM assay constituents and HPLC parameters/ conditions for sample analysis.

Substrate	EFV (CYP2B6)	RIF (esterases)
Concentration (K_m)	15 µM	48 µM
Structure		
Metabolite	E8H	25ODESRIF
Structure		
Internal Standard	NEO	
Concentration	20 µM	
Structure		
Enzyme concentration	0.5 mg/mL	0.25 mg/mL
NADPH concentration	1.3 mM	1.3 mM
Phosphate buffer	0.2 M	0.2 M

HPLC Column	C-18 Phenomenex-Evo column (150 x 2.6 mm, 3.5 µm)	C-18 Phenomenex Luna column (150 x 4.6 mm, 5 µm)
Flow rate	0.7 mL min^{-1}	0.8 mL min^{-1}
Run Time	10.5 min	11.5 min
Injection	15 μL	15 μL
Mobile Phase *(Gradient)*	Water: Acetonitrile 90:10 (0 min) → 20:80 (5 min) → 5:95 (10 min) → 90:10 (11.5 - 12.5 min)	Water: Methanol 90:10 (0 min) → 20:80 (5 min) → 5:95 (10 min) → 90:10 (11.5 - 12.5 min)
Column Temperature	20 ^{o}C	27 ^{o}C
Wavelength	245 nm	254 nm

3. **Rate of metabolite formation**: The rate of metabolite formed per sample analysed in the HPLC was based on the ratio of area under the curve for E8H and 25ODESRIF to that of the internal standard NEO, in each chromatogram. The standard calibration curve was derived for pure E8H and 25ODESRIF in concentrations ranging from 0-150 µM (spiked with 20 µM NEO) based on the ratio of the peak areas for E8H and 25ODESRIF to that of the internal standard NEO plotted against the concentration of E8H and 25ODESRIF. This was further used to calculate the reaction rate (pmol/mg protein/min) and the kinetics constants (K_m and V_{max}).

2.2.4 Determination of kinetics of EFV and RIF

For determining the kinetics of EFV, various concentrations in the range of 0-125 μM were incubated with HLM for 30 min using the same procedure as in section 2.2.5, and the reactions were terminated using 200 µL of chilled acetonitrile spiked with the internal standard, centrifuged and the top layer of the supernatant was subjected to HPLC analysis. The optimal incubation time (30 min) and the concentration of HLM (0.5 mg/mL protein) for the kinetics study was selected based on the rate of metabolite formation from the time/protein-variant incubations.

For determining the kinetics of RIF, various concentrations in the range of 0-150 μM were incubated with HLM, buffer and the co-factors for 30 min, and the reactions were terminated using 200 µL of chilled acetonitrile spiked with NEO, centrifuged and the top layer of the supernatant was subjected to HPLC analysis. The optimal incubation time was maintained at 30 min and the concentration of HLM at 0.25 mg/mL protein.

Apparent kinetic constants (K_m and V_{max}) were estimated by fitting formation rates of metabolite versus substrate concentrations to appropriate kinetic equations by nonlinear regression analysis

using GraphPad Software. The *in vitro* intrinsic clearance, CL_{int} was calculated as below (eq. 2.2.2) (Ogburn et al., 2010):

$$CL_{int} = V_{max}/K_m \qquad \textit{Eq. (2.2.2)}$$

2.2.5 Inhibition assays – HLM and plant extracts

2.2.5.1 Two-point screening

The inhibitory potential of each extract was assessed using a two-point screening with HLM, maintaining the final concentrations of each extract at 20 and 200 µg/mL. The activity potential of each extract was compared with the control incubation of HLM. Based on the K_m values determined, 15 µM EFV and 48 µM RIF were used as substrates for CYP2B6 and the formation of 25ODESRIF (B-esterases), respectively, with 20 µM NEO as the internal standard (Ward et al., 2003). This basic screening was done to analyse if the extracts showed any inhibitory activity. Briefly, a standard 200 µL incubation mixture containing liver microsomes and substrate (0.5 mg/mL protein concentration for 15 µM efavirenz and 0.25 mg/mL protein concentration for 48 µM rifampicin) in 0.2 M phosphate buffer (pH 7.4) and the plant extract (final concentration 20, 200 µg/mL, dissolved in < 1% solvent) at 37 °C was incubated for 30 min, in duplicates. The reactions were initiated with the mix comprising of NADPH (final concentration 1.3 mM) along with the co-factors magnesium chloride (final concentration 3.3 mM), glucose-6-phosphate (final concentration 1.3 mM) and glucose-6-phosphate dehydrogenase (1 U/mL – dissolved in 5 mM sodium citrate buffer), and terminated with the addition of 200 µL of chilled acetonitrile spiked with NEO. The samples were centrifuged using 0.22 µm polypropylene centrifuge tube filtersat 13,000 rpm for 10 min and the supernatants were subjected to HPLC analysis, using same methodology as described in section 2.2.5. TICL (10 and 75 µM) was used as the standard inhibitor control for CYP2B6 (Flockhart, 2007). NELF (10 and 75 µM) was used to in the rifampicin metabolism assays to evaluate if any inhibition could be observed on its metabolism pathway (Polsky-Fisher et al., 2006).

Enzyme activity in the control incubate (no inhibitor), as well as solvent vehicle controls were also measured, in duplicate assays.

The amount of metabolite formed relative to the control was expressed in percentage remaining activity and calculated as in equation (2.2.3):

$$\%\ \text{residual activity} = (\text{Test} - \text{test control})/(\text{Control} - \text{control blank}) \times 100\% \qquad \textit{Eq. (2.2.3)}$$

2.2.5.2 **Assays for determination of IC_{50}**

For the IC_{50} screening of the active extracts, assays were done using the same methodology as in section 2.2.7.1, with concentration range from 1-200 µg/mL of the extract (six-point screening), in triplicates. The assays were repeated to verify inter-day variability and consistency. The reference inhibitors TICL and NELF were screened in the concentration range 1-100 µM, to determine their IC_{50}s. The percentile of residual activity was calculated relative to the test control (no inhibitor), and plotted against the log-transformed concentrations of the herbal extract or the positive control. Non-linear regression analysis (dose-response inhibition) was done to obtain sigmoidal plots of enzyme kinetic data for IC_{50}, using GraphPad Software. The IC_{50} graphs demonstrate the IC_{50} value (μM) calculated using non-linear regression (dose-response inhibition equations) with the actual IC_{50} plot curve-fit based on the concentration of the substrate (0-200 μM and not the log(X) transformation) (Thomford et al., 2016; Awortwe et al., 2014).

2.2.5.3 **Time-dependent inhibition of CYP – Determination of TDI IC_{50} fold-shift**

For the TDI IC_{50} fold-shift assays, the procedure was similar to the IC_{50} screening as in section 2.2.7.2. The plant extracts were pre-incubated with HLM and the co-factors for 30 min prior to addition of the substrate.

All active extracts with concentration range from 1-200 µg/mL (six-point screening) were added to the enzyme/phosphate buffer reaction mixture consisting of phosphate buffer (0.2 M), final protein concentration of 0.5 mg/mL for EFV and 0.25 mg/mL for RIF and 90 µL of the same was mixed with 100 µL of reaction mix comprising of glucose 6-phosphate dehydrogenase (1 U/mL – dissolved in 5 mM sodium citrate buffer), and cofactors (1.3 mM NADPH, 3.3 mM $MgCl_2$, 1.3 mM glucose 6-phosphate) in test-tubes. The concentration of HLM was similar to that used in the IC_{50} assay. The tubes were pre-incubated at 37 °C for 30 min followed by addition of 10 µL of substrate reaction mixture of 15 µM EFV (CYP2B6) and 48 µM RIF, in phosphate buffer (0.2 M) to each tube and incubated for additional 30 min, along with the test control tubes (no inhibitor). The reaction was terminated using chilled ice-cold acetonitrile spiked with 20 µM NEO and the samples were centrifuged using 0.22 µm polypropylene centrifuge tube filtersat 13000 rpm for 5 min, and the supernatants were subjected to HPLC analysis. The percentile of residual activity was calculated relative to the test control (no inhibitor), and plotted against the log-transformed concentrations of the herbal extract or the positive control. Non-linear regression analysis was done to obtain sigmoidal plots of enzyme kinetic data for the fold shift in IC_{50} for each herbal extract. The TDI fold-shift was determined

for each herbal extract based on the ratio of the IC_{50}s of the co-incubation assay IC_{50}(-) to that of the pre-incubation assay IC_{50}(+), with NADPH (equation. 2.2.4).

$TDI_{fold\text{-}shift}$ = co-incubation assay IC_{50}(-)/ pre-incubation assayIC_{50}(+) *Eq. (2.2.4)*

Extracts with fold shifts ≥1.5 were classified as positive for TDI (Nomeir et al., 2004; Nebert and Russell, 2002).

PHASE – III: CYP INDUCTION STUDY

2.2.6 Induction assays – mRNA expression in hepatocytes

2.2.6.1 Cytotoxicity testing

In vitro cytotoxicity of the plant extracts were tested against HepG2 (human liver carcinoma) cell line. The plant extracts were taken at concentrations ranging from 1000.00-31.25 µg/mL to determine their percentage growth inhibition and toxic concentrations on HepG2 cell line (Nagarajappa et al., 2016; Denizot and Lang, 1986).

2.2.6.1.1 Preparation of herb extract test solution

For cytotoxicity studies, 100 µL of each plant extract was separately suspended and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 10% $^{w}/_{v}$ concentration, and filtered using 0.22 µm syringe filter. Serial two fold dilutions were prepared from this for carrying out the cytotoxicity studies.

2.2.6.1.2 Cell line and culture medium

HepG2 cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 I U/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96-well microtiter plates (Freimoser et al., 1999).

2.2.6.1.3 Cytotoxicity testing and determination of CTC_{50}

Cytotoxicity of the plant extracts was assessed based on the method described in a previous study on *Coleus forskohlii* (Nagarajappa et al., 2016). The HepG2 monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/mL using DMEM containing 10% FBS. To each well of the 96-well microtitre plate, 0.1 mL of the diluted cell suspension was

added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer washed once with the medium and 100 µL of different test concentrations of the herbal extracts were added on to the partial monolayer in the microplates. The plates were then incubated at 37 °C for 72 h in 5% CO_2 atmosphere, and microscopic examination was carried out; observations were noted every 24 h. After 72 h, the extract solutions in the wells were discarded and 50 µL of tetrazolium dye (MTT) in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C in 5% CO_2 atmosphere. The supernatant was removed and 100 µL of iso-propanol was added and the plates were gently shaken to solubilize the formazan crystals formed. Finally, the absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the standard formula:

%growth inhibition = (Control absorbance–test absorbance)/Control absorbance$^{\times}$100 *Eq. (2.2.4)*

From the dose-response curves for each extract against the cell lines, the concentration of the test extract needed to inhibit cell growth by 50% (CTC_{50}) was generated (Nagarajappa et al., 2016).

2.2.6.2 **Plant sample analysis - extraction and purification of total mRNA**

2.2.6.2.1 **Extraction and purification of total mRNA**

The mRNA expression levels of CYP3A4 and CYP2B6 were carried out using semi-quantitative RT-PCR. Briefly, the HepG2 cells were cultured in 60 mm petridishes and maintained in DMEM medium for 48 h. The DMEM medium was supplemented with FBS and amphotericin. Each plant extract (CTC_{50} concentration) was added to a petridish comprising of the cells and incubated for 24 h. Total cellular RNA was isolated from the untreated (control) and treated cells using Tri-Xtract™ according to the protocol described by the manufacturer (G-Biosciences Ltd). The mRNA pellet was dissolved in nuclease-free water. Purity of RNA was estimated using the 260/280 absorbance ratio, and samples were frozen at -80 °C for further analysis.

2.2.6.2.2 **RT-PCR Procedure**

cDNA was synthesized from the total isolated RNA from the treated HepG2 cells, by reverse transcriptase kit according to the manufacturer's instructions (Thermo Scientific). The reaction mixture consisted of 1x cDNA synthesis buffer, dithiothreitol (0.5 M), RiboLock RNAse inhibitor (20 U), deoxynucleotide mix (1.6 mM), oligo dT (100 ng), reverse transcriptase (25

U), and total RNA. Primers for CYP3A4 and CYP2B6 were selected as per a method developed previously for studying the modulation of CYPs (Park et al., 2009). 50 µL of the reaction mixture was subjected to PCR for amplification of hepatic cells. cDNAs using specifically designed primers (procured from Eurofins, India) were used and as an internal control, the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was co-amplified with each reaction. Dexamethasone (10 µM) and rifampicin (50 µM) were used as positive controls for CYP2B6 and CYP3A4, respectively (Nagarajappa et al., 2016).

2.2.6.2.3 **Amplification conditions for pro-apoptotic gene**

CYP3A4: Polymerase chain reaction conditions were set for initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing of primers at 62 °C for 30 seconds and extension at 72 °C for 45 seconds. This was followed by final extension at 72 °C for 10 min.

For the first strand synthesis, oligo dT primer was used and for second strand synthesis, 5' ATTCAGCAAGAAGAACAAGGACA 3' and 5' TGGTGTTCTCAGGCACAGAT 3' were used as the forward and reverse primers, respectively (Product size: 314 bp).

CYP2B6: Polymerase chain reaction conditions were set for initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 15 seconds, annealing of primers at 62 °C for 30 seconds and extension at 72 °C for 45 seconds. This was followed by final extension at 72 °C for 10 min.

For the first strand synthesis, oligo dT primer was used and for second strand synthesis, 5' ATGGGGCACTGAAAAAGACTGA 3' and 5' AGAGGCGGGGACACTGAATGAC 3' were used as the forward and reverse primers, respectively (Product size: 283 bp).

2.2.6.2.4 **Analysis of amplified sequences**

The amplified samples were analysed through 1.8% agarose gel electrophoresis. The gel was scanned with UV illumination using digital imaging, and relative sample expression levels were calculated using Syngene inGenius documentation system and GeneTools analysis software.

2.2.6.2.5 **Statistical analysis**

Experimental data were expressed as the mean ± standard deviation or standard error of mean. The results were subjected to one-way analysis of variance, followed by Dunnett's multiple comparison tests, by fixing the significance level at $p < 0.05$, $p < 0.01$ and $p < 0.001$

(Nagarajappa et al., 2016). The statistical analyses were performed using GraphPad Prism Version 5.0.

PHASE – IV: PHYTOCHEMICAL FINGERPRINTING

2.2.7 Preparation of extracts

About 8-10 mg of each extract was weighed in an Eppendorf tube and 1 mL of 50% methanol in water containing 2% formic acid was added, followed by dissolution in an ultrasonic bath (0.5 Hz, Integral Systems, RSA) for 20 min at room temperature. The extracts were centrifuged (Hermle Z160m, 3000 g for 5 min) and transferred to vials.

2.2.8 Preparation of standards

The stock solutions of the reference standards quercetin and gallic acid were prepared in 1000 mg/L concentrations in 50 mL volumetric flasks using 50% methanol in water containing 2% formic acid; 4 mL of DMSO was used to dissolve quercetin completely before the solution was made up to 50 mL in the volumetric flask. Using both stock solutions a cocktail was prepared in the concentration of 200 mg/L of each standard, to enable isomers and compounds with similar elemental formulas to be differentiated.

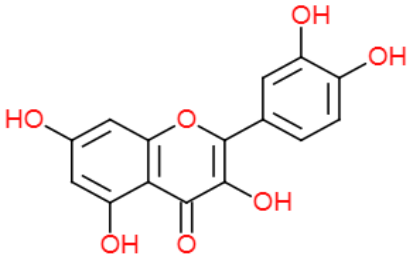
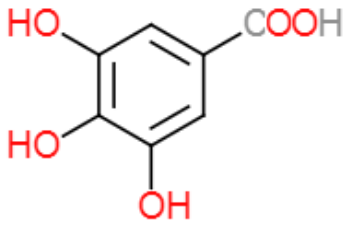
2.2.9 LC-MS analysis conditions: Phytochemical fingerprinting

Phytochemical fingerprinting of the extracts was done using high-resolution UPLC-MS analysis. A Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) was used for the same. Electrospray ionization was used in both negative and positive modes with a cone voltage of 15 V, desolvation temperature of 275 °C, desolvation gas at 650 L/h. The MS settings were optimized for best resolution and sensitivity (Stander et al., 2017). Data were acquired by scanning all extracts, from 150 to 1500 *m/z* in resolution mode as well as in MS^E mode. In MS^E mode two channels of MS data were acquired, one at a low collision energy (4 V) and the second using collision energy ramp (40−100 V) to obtain fragmentation data as well. The UPLC-MS was calibrated with sodium formate and leucine enkaphalin was used as reference mass (lock mass) for accuracy in the mass determination. A Waters HSS T3, 2.1 × 100 mm, 1.7 µm column was used for the separation. 2 µL of each extract was injected, and a mobile phase comprising of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B, was used. A gradient run was used with a flow rate of 0.3 mL min^{-1}, starting with 100% solvent A for 1 min, which was changed to 28%

B over 22 min in a linear way. The gradient solvent composition was then changed to 40% B over 50 seconds followed by a wash step with 100% solvent B for 1.5 min, and finally re-equilibration (to initial conditions) for 4 min. The column temperature was maintained at 55 °C. The PDA wavelength range was set between 230 and 600 nm.

The reference standards quercetin and gallic acid along with the plant extracts were analysed as per the specific LC-MS/ PDA parameters/ conditions as outlined below in Table 4.

Table [4] LC-MS/PDA parameters of the phyto-profiling method.

Column	Waters HSS T3, 2.1 × 100 mm, 1.7 µm	
Phytocompound	Flavonoids	Phenols
Reference Standard	Quercetin (Ramos et al., 2017)	Gallic Acid (Vallverdú-Queralt et al., 2015)
Structure		
Flow rate	0.3 mL min^{-1}	
Run Time	29 min for negative mode run, 15 min for positive mode run.	
Injection	2 µL	
Mobile Phase	0.1% Formic Acid in Water : 0.1% Formic Acid in Acetonitrile *Gradient run*	
Column Temperature	55 ºC	
PDA Wavelength	230 – 600 nm	

The methods were tested for accuracy and linearity. A linear response was obtained for quercetin for the positive mode runs, in the range of 200.000-6.250 mg/L (R^2 = 0.9900) (Fig. 13). This concentration range was selected to identify and compare the peak-retention factors and *m/z* of the herbal extract with the reference standard, along with reasonable approximations of the relative amounts of the identified peaks from the herbal extracts using the standard calibration curve of quercetin. For all the negative scans, both quercetin and gallic acid were used as reference standards to relatively quantify the peaks for major compounds with each extract. The calibration curve showed a slight bend (non-linearity) at higher concentrations (200 mg/L) of both standards in the negative mode. A quadratic linear curve fitting model was used for quercetin (R^2 = 0.9879) for relative quantification of the unknown phytocompounds, based on the area peak area (response) for the concentration range used (200.000-6.250 mg/L)

whereas a linear fit model was used for gallic acid (R^2 = 0.9680) since the peak area (response) was less for low concentrations.

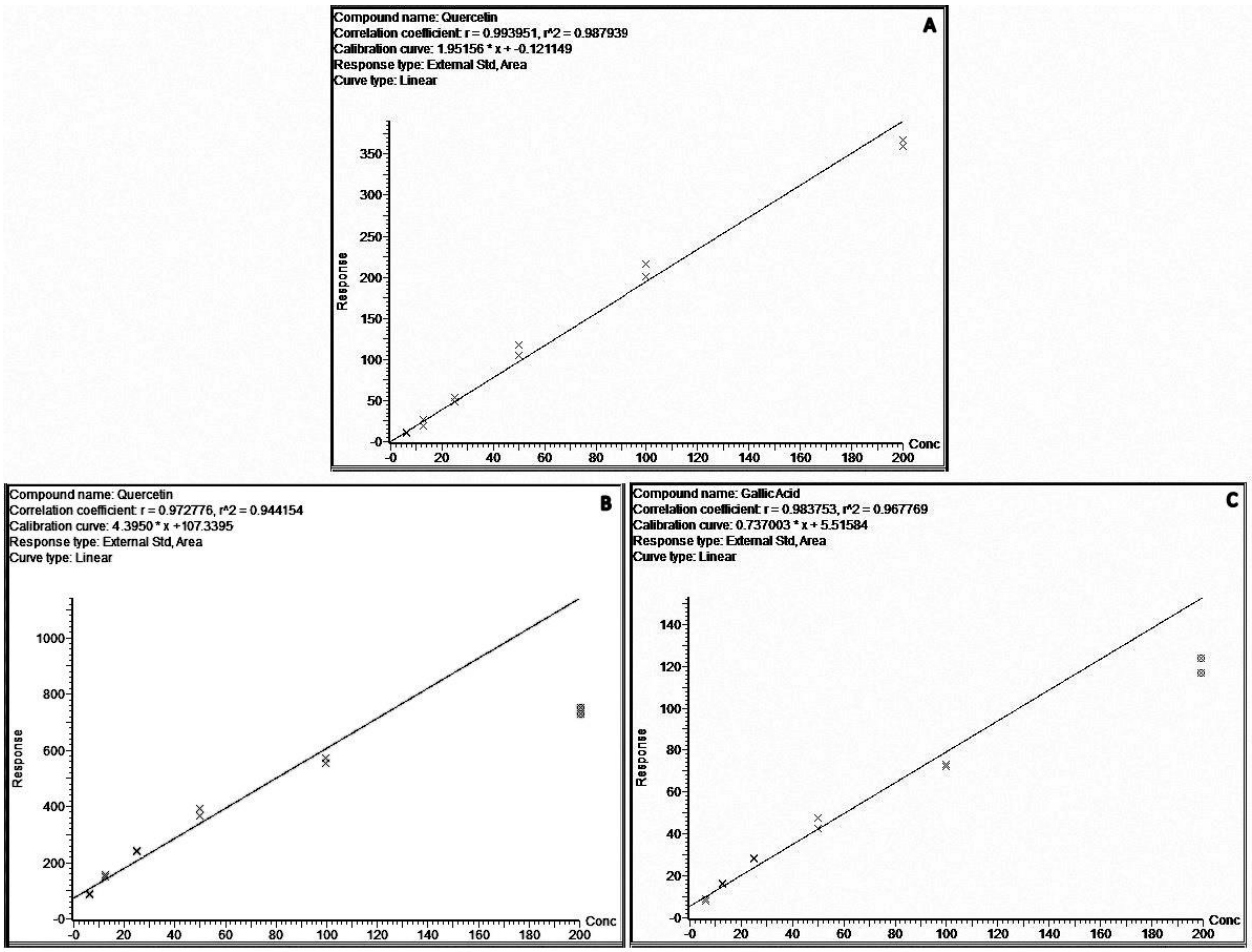


Fig. 13–Concentration range linearity for quercetin in positive mode scan, **B** &**C** –regression line-fit concentration range linearity for gallic acid & quercetin in negative mode scans, respectively (LC-MS).

Tentative identification of the possible phytocompounds was derived based on:

- Accurate masses
- *m/z* transitions (MS/MS fragments)
- UV maxima
- Relative retention times and comparison with literature review on matching compounds
- Online mass spectral repositories:
  - ✓ Metlin Scripps (*https://metlin.scripps.edu/*)
  - ✓ NIST Standard Reference Data online Webbook library (*http://webbook.nist.gov/chemistry/mw-ser.html*)
  - ✓ MassBank online Spectral Database (*https://massbank.eu/MassBank/*)
  - ✓ Pubchem chemistry database (*https://pubchem.ncbi.nlm.nih.gov*).

Chapter 3
RESULTS

3.1 Solvent extraction yield of herbs

The calculated yields (per 4 g of dried herb) of the solvent extracts of the investigated herbs are shown in table 5. Highest yields were observed in the aqueous solvent extractions. *Astragalus membranaceus* had relatively high yields per solvent extracts, and had highest yield among all ethanol extracts. *Inula helenium* had the highest yield among the aqueous extracts, while *Glycyrrhiza glabra* had the highest yield among all the methanol extracts, with 27.36%. *Althaea officinalis* had the highest yield among all ethyl acetate extracts with 14.37%.

Table [5] Extraction yield of herbs.

Herb	Yield ($^{\pm}$mg, % $^{w}/_{w}$)			
	Aqueous Extract (A1)	**Methanol Extract (M2)**	**Ethanol Extract (E3)**	**Ethyl acetate Extract (EtA4)**
Withania somnifera (Ws)	$^{\pm}$443 mg, 11.08%	$^{\pm}$489 mg, 12.22%	$^{\pm}$237 mg, 5.92%	$^{\pm}$30 mg, 0.75%
Glycyrrhiza glabra (Gg)	$^{\pm}$364 mg, 9.10%	$^{\pm}$1094 mg, 27.36%	$^{\pm}$430 mg, 10.75%	$^{\pm}$47 mg, 1.18%
Astragalus membranaceus (Am)	$^{\pm}$1147 mg, 28.69%	$^{\pm}$803 mg, 20.09%	$^{\pm}$473 mg, 11.82%	$^{\pm}$195 mg, 4.88%
Inula helenium (Ih)	$^{\pm}$2198 mg, 54.95%	$^{\pm}$18 mg, 0.44%	$^{\pm}$295 mg, 7.37%	$^{\pm}$262 mg, 6.54%
Althaea officinalis (Ao)	$^{\pm}$1167 mg, 29.16%	$^{\pm}$731 mg, 18.29%	$^{\pm}$426 mg, 10.66%	$^{\pm}$575 mg, 14.37%
Ocimum basilicum (Ob)	$^{\pm}$1343 mg, 33.57%	$^{\pm}$378 mg, 9.44%	$^{\pm}$118 mg, 2.94%	$^{\pm}$193 mg, 4.83%

$^{\pm}$**mg -** ***approximate,*** considering the residual amount of extract retained in the glass tubes after scraping.

3.2 Biochemical analysis of herbs

Based on the color intensity and amount of precipitate formed, the biochemical tests confirmed the presence of phytoconstituents such as alkaloids, glycosides, terpenoids, phenols, coumarins and flavonoids within most extracts (Table 6). The following observations were noted:

- All investigated phytocompounds, except steroids could be detected in *Glycyrrhiza glabra* extracts, coumarins being most prominent.
- Coumarins, alkaloids and flavonoids were present in all the extracts of *Inula helenium*.
- *Astragalus membranaceus* extracts contained all phytocompounds except steroids and tannins. The intensity of precipitate formation for flavonoids was high in the aqueous, methanolic and ethanolic extracts.

Table [6] Biochemical Phyto-profiling. **Legend**✓ Present, ++ Intensity, --- Absent

Sl #	Phyto Constituent	Test	Result (positive)	Extract	Inference					
					Ws	***Gg***	***Am***	***Ih***	***Ao***	***Ob***
1 *a.*	Alkaloids	Harborne Test	Yellow coloration in extract indicates the presence of alkaloids.	A1	✓	✓+++	✓+++	✓+++	✓+++	✓++
				M2	✓+++	✓+++	✓+	✓+++	✓+	✓+++
				E3	✓+++	✓+++	✓+++	✓+++	✓+	✓++
				EtA4	✓+	✓+++	✓+	✓+++	✓+	✓++
b.		Wager's Test	Reddish-brown color formation indicates positive.	A1	✓+++	---	✓	✓+++	---	✓+++
				M2	✓+++	✓+++	✓+++	✓+++	✓+++	✓+++
				E3	✓+++	✓+++	✓+++	✓+++	✓+++	✓+++
				EtA4	✓+++	✓+++	✓+++	✓++	✓++	✓+
2	Saponins	Emulsion test	Formation of emulsion, which remains consistent.	A1	✓+++	✓+++	✓+	✓+++	---	✓+++
				M2	---	✓+++	✓+++	✓+	---	✓+++
				E3	---	---	---	---	---	✓+++
				EtA4	---	---	✓+	---	---	✓++
3	Phenols	Ferric Chloride Test	Greenish colour was observed in extract.	A1	---	✓+++	✓	---	✓	✓+++
				M2	---	✓+++	✓+++	---	✓+	✓+++
				E3	---	✓+++	✓	---	---	✓
				EtA4	---	✓+	✓	---	---	✓
4	Tannins	Harborne Test	Blue-black color formation indicates presence of Tannin.	A1	---	✓+++	---	---	✓+++	✓+++
				M2	---	✓+++	---	---	✓+	✓+++
				E3	---	✓+++	---	---	---	✓+
				EtA4	---	✓+	---	---	---	✓
5	Glycosides	Keller-Kiliani Test	Brown ring colour formation tests positive.	A1	✓+	✓+++	✓	✓+++	✓	✓
				M2	✓+++	✓+++	✓+++	✓+++	✓+++	✓+++
				E3	✓++	✓+++	✓+++	✓+++	✓+++	✓+++
				EtA4	✓++	✓++	✓+++	✓+	✓++	✓
6	Terpenoids	Salkowski Test	A reddish brown colouration if observed indicates positive.	A1	✓++	✓+++	✓	✓+++	✓+	✓+++
				M2	✓+++	✓+++	✓++	✓+++	✓++	✓+++
				E3	✓+++	✓+++	✓++	✓+++	✓++	✓+++
				EtA4	✓+	✓++	✓	✓+	✓++	✓+
7	Flavonoids	Vanillin Test	A reddish brown coloured precipitate is formed.	A1	---	✓+	✓+++	✓+	---	---
				M2	✓+++	✓++	✓+++	✓+++	✓+++	✓+++
				E3	✓++	✓++	✓+++	✓++	✓+++	✓+++
				EtA4	✓+	✓++	✓+	✓+	✓++	✓+
8	Steroids	Liebermann-Burchard Test	Dark bluish precipitate indicates the presence.	A1	✓	---	---	---	---	---
				M2	✓	---	---	✓+	---	---
				E3	✓	---	---	✓	---	✓
				EtA4	---	---	---	---	---	✓
9	Coumarins	Sodium Hydroxide Test	Formation of yellow colour indicates presence.	A1	✓+	✓+++	✓+++	✓+++	✓+++	✓
				M2	✓+++	✓+++	✓+++	✓+++	✓+++	---
				E3	✓++	✓+++	✓++	✓+++	✓+	---
				EtA4	✓+	✓+++	✓+	✓+++	✓	✓

- In the extracts of *Withania somnifera* mostly glycosides, alkaloids and phytosteroids were observed.
- Tests on the extracts of *Ocimum basilicum* showed positive for all compounds, coumarins and phytosteroids being present in trace amounts.
- The extracts of *Althaea officinalis* prominently had only alkaloids, glycosides and flavonoids.

According to this qualitative analysis *G.glabra*, *O.basilicum*, *I.helenium* and *A.membranaceus* contained most of the relevant groups of phytocompounds. *W.somnifera* and *A.officinalis* had least saponins, tannins and phenols.

3.3 Inhibition assays

3.3.1 HLM assay validation

Satisfactory separations of all three analytes EFV, E8H and NEO, per assay were not achieved when using an isocratic method, especially due to interference from co-factors and secondary metabolites in the HLM assays; hence, gradient elution was selected (Varghese et al., 2014). Method optimization was done by modifying the run conditions such as change in column length (100 mm, 150 mm and 250 mm), inner diameter (2.6 mm and 4.6 mm), particle size (3.5 µm and 5 µm), and change in the elution to gradient.

1. **HPLC–EFV methods**: Satisfactory separations were achieved on the C-18 column (150 x 4.6 mm, 2.6 µm), by gradient elution using mobile phase comprising of water (A): acetonitrile (B) at a flow of 0.7 mL min^{-1}. Comparative analysis was done using a biphenyl column of the same dimension and 5µmparticle size (Restek Corp., USA) (Fig. 15). However compared to the separation achieved in the biphenyl column (Fig. 15), the HPLC retention times were consistent at ±7.57 min, ±8.15 min, and ±8.77 min for EFV, E8H and NEO, respectively for the C-18 column with a shorter run time (Fig. 14).

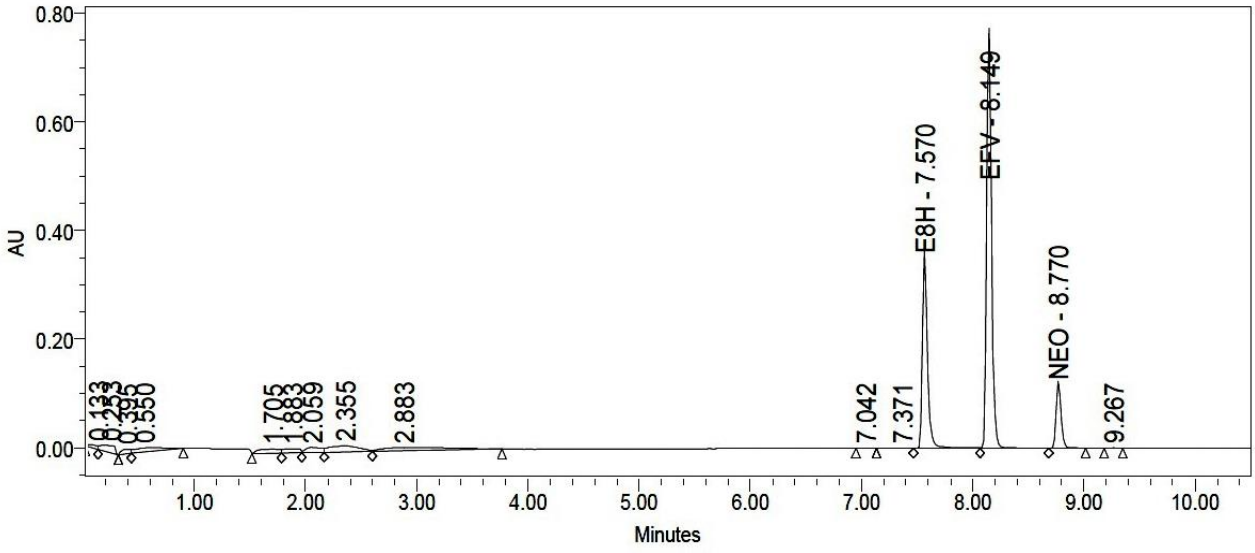


Fig.14 HPLC chromatographic separation of E8H, EFV and NEO using the C-18 column at 200 μM concentrations of all the drugs.

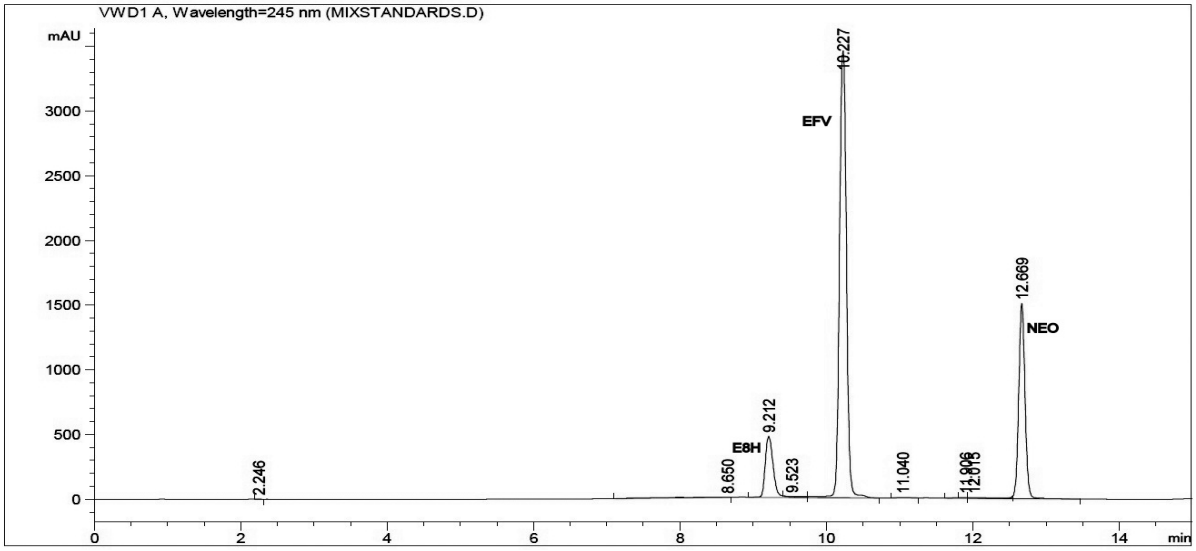


Fig.15 HPLC chromatographic separation of E8H, EFV and NEO using the biphenyl column at 4 mM concentrations of all the drugs.

A linear response was obtained in the concentration range 0 – 200 μM for both EFV and E8H (R^2 = 0.9930). LLOD and LLOQ were calculated at 7.57 μM and 22.95 μM for EFV and 7.99 μM and 24.24 μM for E8H, respectively (Table 13).

The HPLC method was also validated using *in vitro* human liver microsomal incubation assay. For all four incubation times (15, 30, 45 and 60 min), the metabolite E8H was detected using this method at consistent retention times along with EFV and NEO, and the peak area of NEO was relatively constant. Linearity was established for the 15-60 min incubation range based on the ratio of the metabolite to the internal standard, with R^2 = 0.9934 (Fig. 17a, Table 7).

2. **HPLC–RIF method**: A linear response was obtained in the concentration range 0 – 200 μM for both RIF and 25ODESRIF (R^2 = 0.9950). LLOD and LLOQ were calculated at 5.86 μM and 17.75 μM for RIF and 7.78 μM and 23.57 μM for 25ODESRIF, respectively (Fig. 17b, Table 7). The results of the system suitability tests assure the adequacy of the proposed HPLC method for routine analysis of RIF and 25ODESRIF alone or in combination (Fig. 16. 1).

In the *in vitro* human liver microsomal incubation assays, for all four incubation times (15, 30, 45 and 60 min, in duplicates) the metabolite 25ODESRIF was detected using this method at consistent retention times along with RIF and NEO, and the peak area of NEO was relatively constant (Fig. 16. 2). Linearity was established for the 15-60 min incubation range based on the ratio of the metabolite to the internal standard, with R^2 = 0.9901 (Fig. 17. a-b, Table 7).

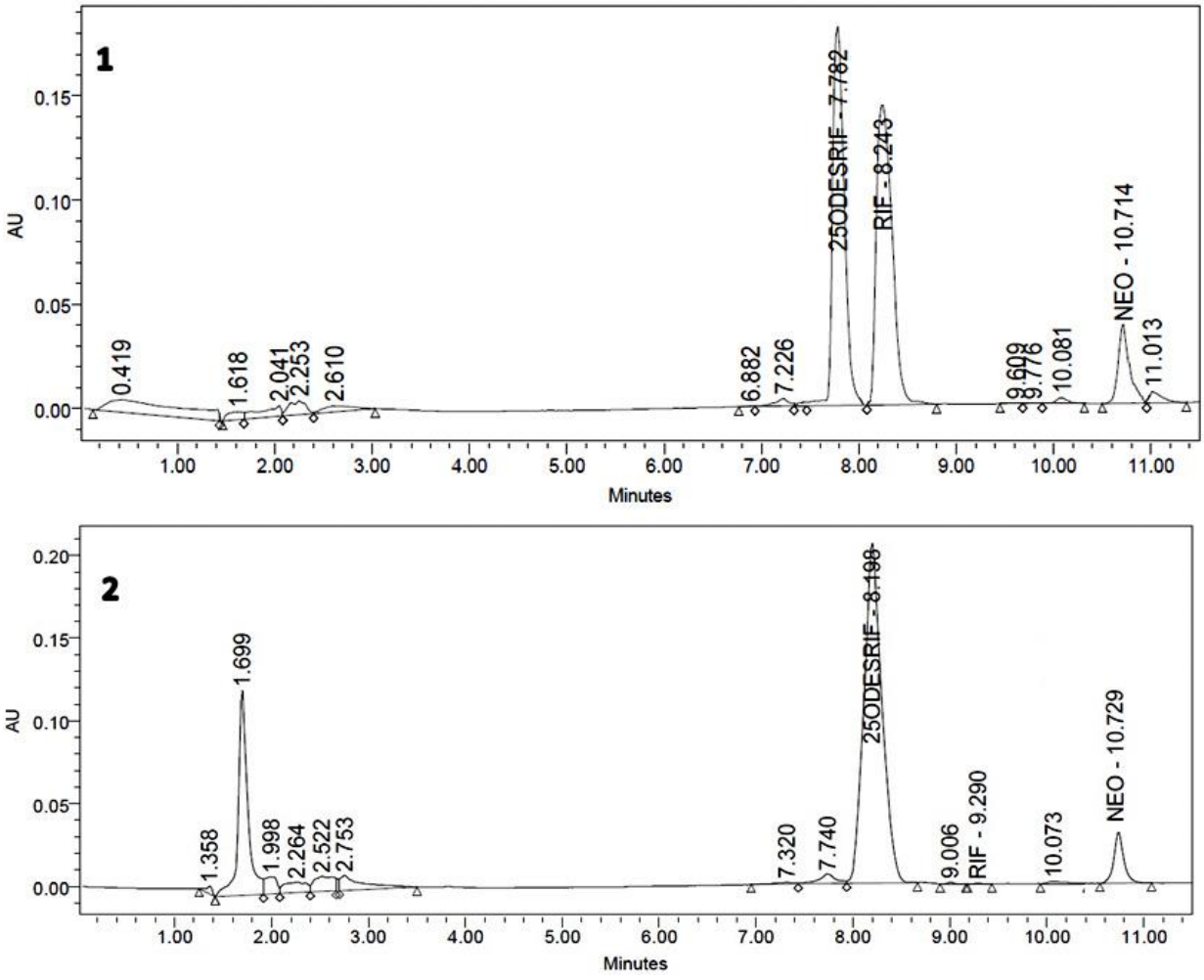


Fig.16 1.HPLC chromatographic separation of RIF, 25ODESRIF and NEO using the C-18 Luna column at 100 μM concentrations of the drug and its metabolite, 20 μM internal standard, 2. HPLC chromatographic separation of RIF, 25ODESRIF and NEO using the C-18 Luna column, for the *in vitro* HLM-RIF incubation sample (75 μM RIF)

Table [7] HPLC analysis retention time for both methods.

Column	Phenomenex-Evo C-18 100A Column (150 x 4.6 mm, 2.6 µm)			Phenomenex Luna C-18 Column (150 x 4.6 mm, 5 µm)		
Drug	EFV	E8H	NEO	RIF	25ODESRIF	NEO
Retention Time (*min*)	7.57	8.15	8.77	7.70	8.25	10.70
LLOD (*µM*)	7.57	7.99	-	5.86	7.78	-
LLOQ (*µM*)	22.95	24.24	-	17.75	23.57	-
Linear Correlation Coefficient (R^2)	0.9906	0.9948	-	0.9932	0.9976	-
Overall Run Time (*min*)	10.5			11.5		
HLM Time-variant assay Linear Correlation Coefficient (R^2)15-60 *min*	0.9934			0.9901		

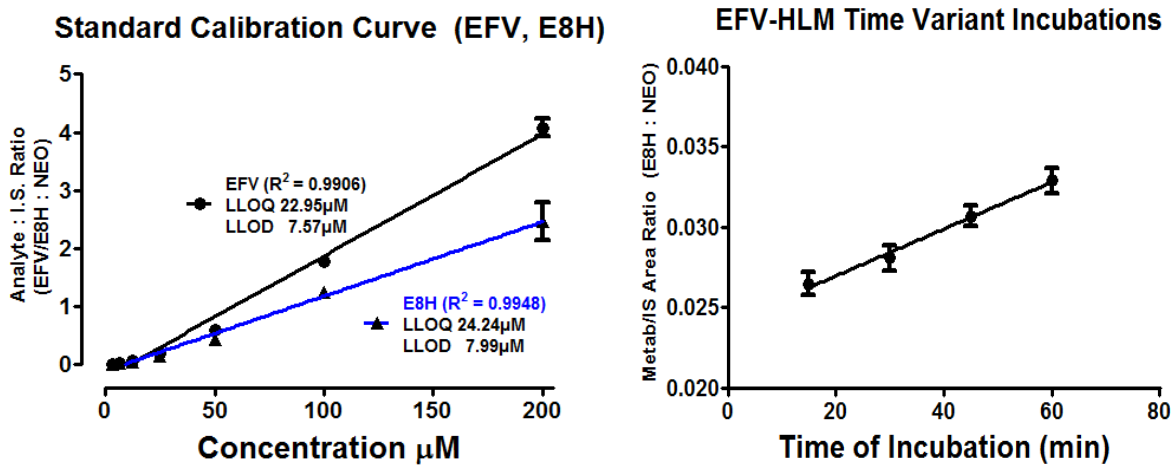


Fig.17a Standard calibration curves, regression statistics for EFV and E8H; HLM time variant incubation assay linearity showing the ratio of E8H to NEO, for the specific time of incubation (15, 30, 45 and 60 min).

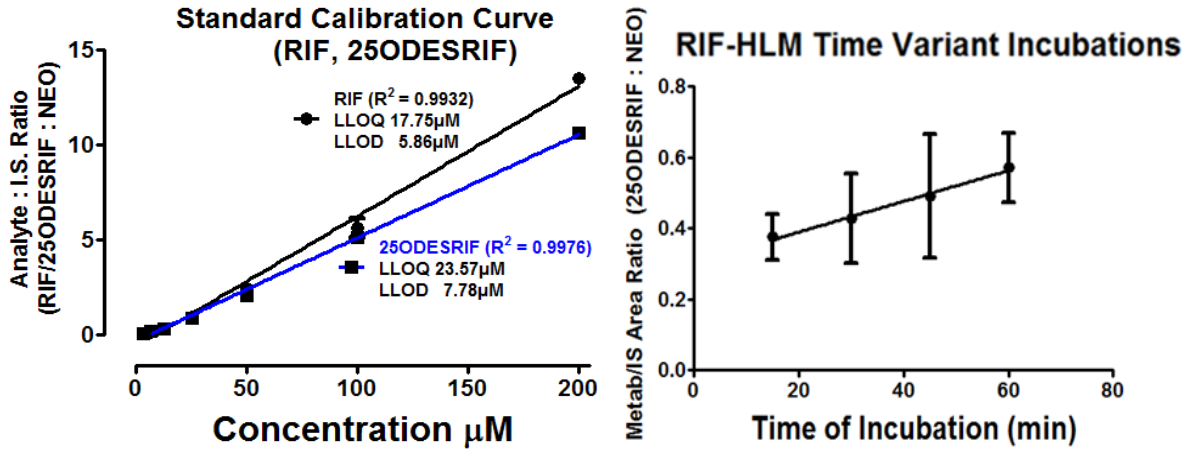


Fig.17b Standard calibration curves, regression statistics for RIF and 25ODESRIF; HLM time variant incubation assay linearity showing the ratio of 25ODESRIF to NEO, for the specific time of incubation (15, 30, 45 and 60 min).

3.3.2 Kinetic analyses of EFV, RIF and their metabolites

The kinetics for the formation of E8H from EFV, and 25ODESRIF from RIF were determined in several HLM incubations for concentrations in the range 0 – 150 μM. Representative Michaelis-Menten kinetic plots from all assays are depicted in Fig.18, and the kinetic parameters are summarized in Table 8.

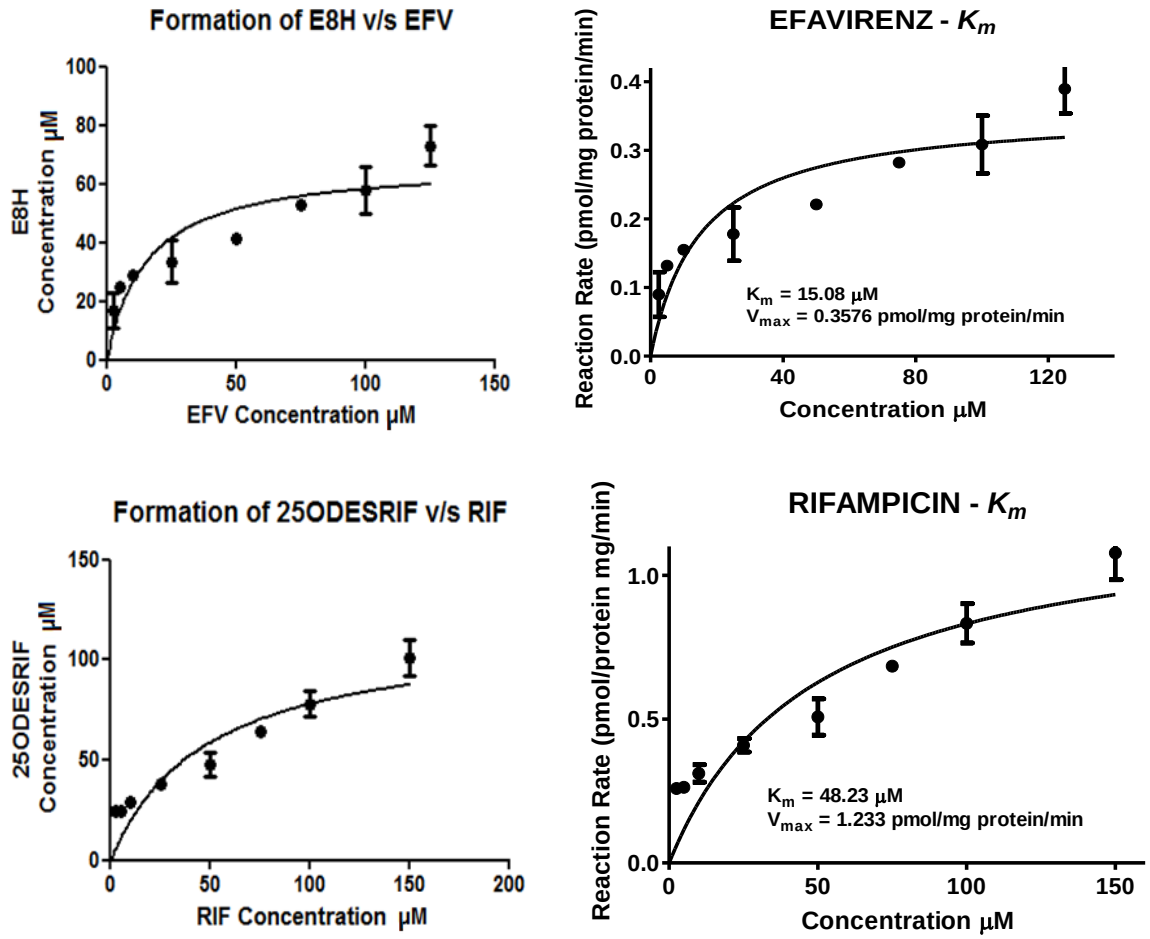


Fig.18 Kinetic analyses of EFV and RIF by human liver microsomes, and formation of E8H versus EFV and 25ODESRIF versus RIF.

Table [8] Kinetics of efavirenz and rifampicin metabolism.

HLMs	Enzyme	SUBSTRATE	K_m	V_{max}	CL_{int} (V_{max}/ K_m)
H0610, H0620, H0630, H0640 *(Pooled HLM – Mixed, Xenotech)*	2B6	EFV	15.08	0.3576	0.0240
	B-esterases	RIF	48.23	1.2330	0.0260

V_{max}, pmol/min/mg protein or pmol/min/pmol P450; K_m, μM; CL_{int}, μl/min/mg protein or μl/min/pmol P450.

Efavirenz with its binding affinity to CYP2B6 had K_m and V_{max} values of 15.08 μM and 0.3576 pmol/min/mg protein. The intrinsic clearance CL_{int} was calculated as V_{max}/ K_m and observed to be 0.0240 μl/min/mg protein for EFV.

For rifampicin, the analysed K_m and V_{max} values were 48.23 μM and 1.2330 pmol/min/mg protein, and CL_{int} was calculated as 0.0260 μl/min/mg protein.

3.3.3 Two-point screening of herbs

The potential of the selected herbal extracts to inhibit the enzyme activity of CYP2B6 and the metabolism pathway of rifampicin was investigated. All herbal extracts were screened at two concentrations (20 µg/mL and 200 µg/mL) and the observed remaining activity was expressed as percentages of the negative control (no inhibitor). For both enzymes, less than 1% solvent was used for the total incubation, as per the assay standard guidelines. For the solvent controls (solvent concentration > 1%) ethanol reduced CYP2B6 activity by 20% (illustrated in fig. 21) and methanol inhibited rifampicin metabolism by 20% (illustrated in fig. 24), while ethyl acetate had no effect on both CYP2B6 and rifampicin metabolism. All solvent controls with concentration < 1% had no inhibitory effect on the enzyme activity.

CYP2B6: For the aqueous extracts screened with EFV as substrate (against CYP2B6) *Ocimum basilicum* (ObA1) and *Inula helenium* (IhA1) showed most inhibitory effect. ObA1 reduced CYP2B6 activity by less than 50%, while IhA1 reduced the activity by around 50%. *Glycyrrhiza glabra* (GgA1) extract showed a major increase in the rate of metabolism by more than 200% at 200 μg/mL concentration (Fig. 19).

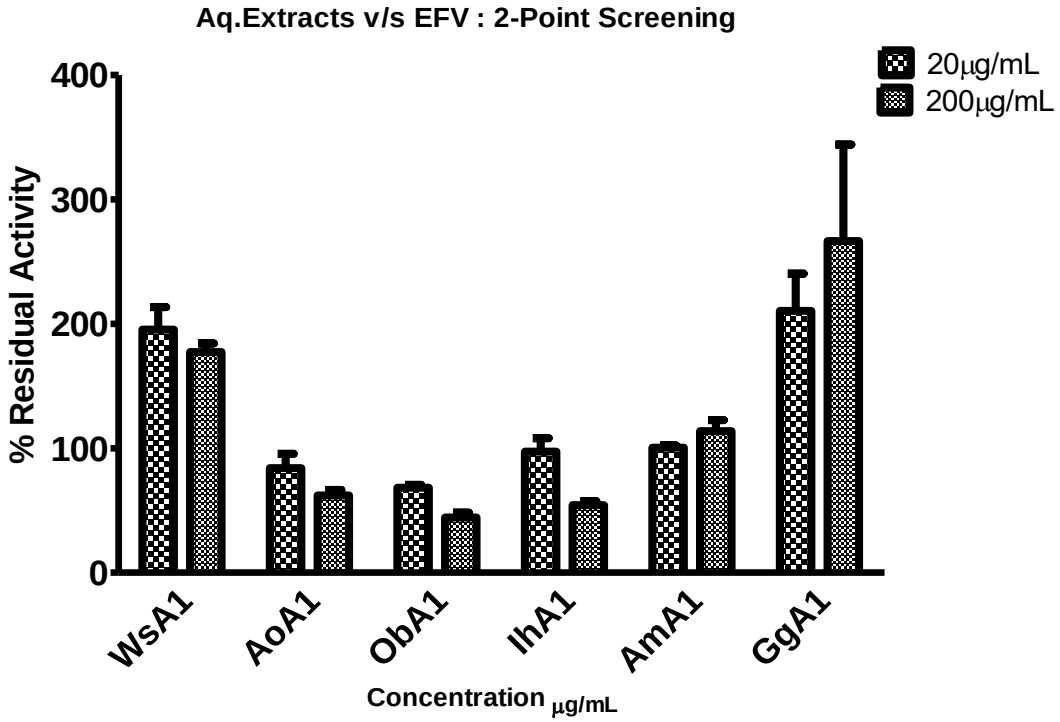


Fig.19 2-Point screening of all aqueous extracts against CYP2B6 (Code name for each herb can be referred to table 2).

For the methanolic extracts screened against CYP2B6, most of the extracts had an inhibitory effect. *Inula helenium* (IhM2) showed the largest inhibitory effect reducing the CYP2B6 activity to around 30% and ObM2 reduced activity by less than 50%, while *Withania somnifera* (WsM2) reduced the activity to 60%. *Glycyrrhiza glabra* (GgM2) extract showed a major increase in the metabolism by more than 200% similar to its aqueous extract (Fig. 20).

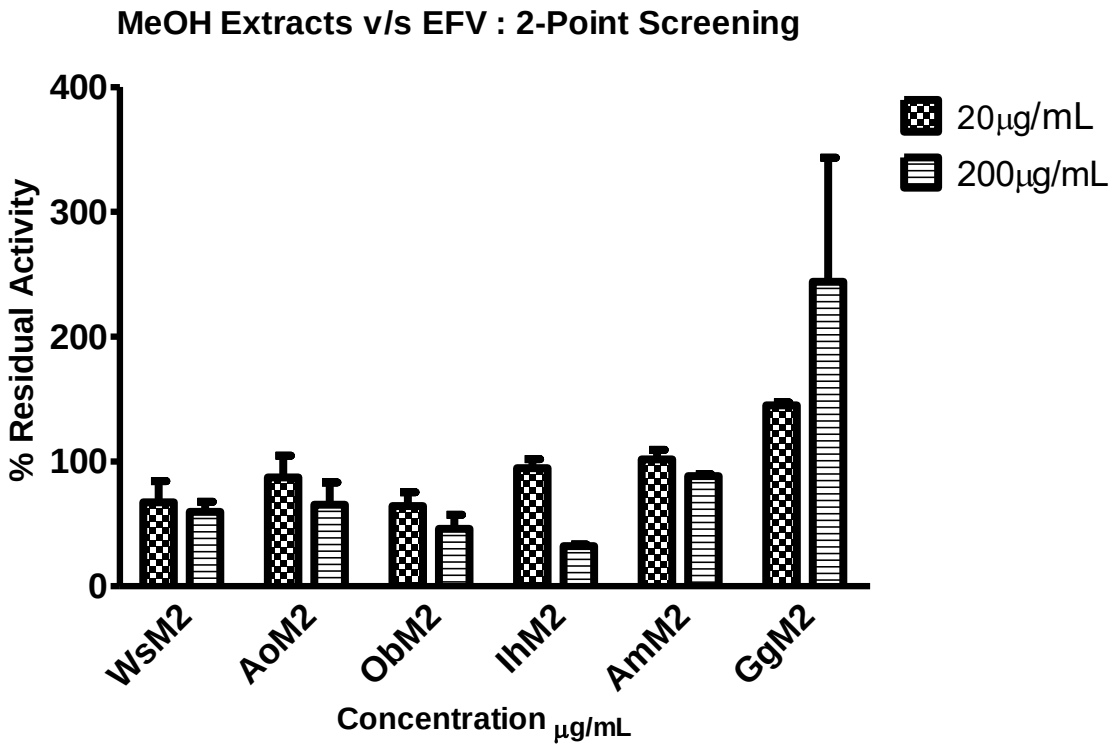


Fig.20 2-Point screening of all methanolic extracts against CYP2B6 (Code name for each herb can be referred to table 2).

Amongst the ethanolic extracts, except for *Glycyrrhiza glabra* (GgE3) all the other extracts inhibited CYP2B6 activity; *Inula helenium* (IhE3) reduced the activity to around 30% and *Astragalus membranaceus* (AmE3) reduced the activity by 50% (Fig. 21).

GgE3 enhanced the rate of EFV metabolism, increasing the activity by 200%. This mechanism of CYP2B6 activation was similar to the trend observed for the aqueous and methanolic extracts of *G.glabra*.

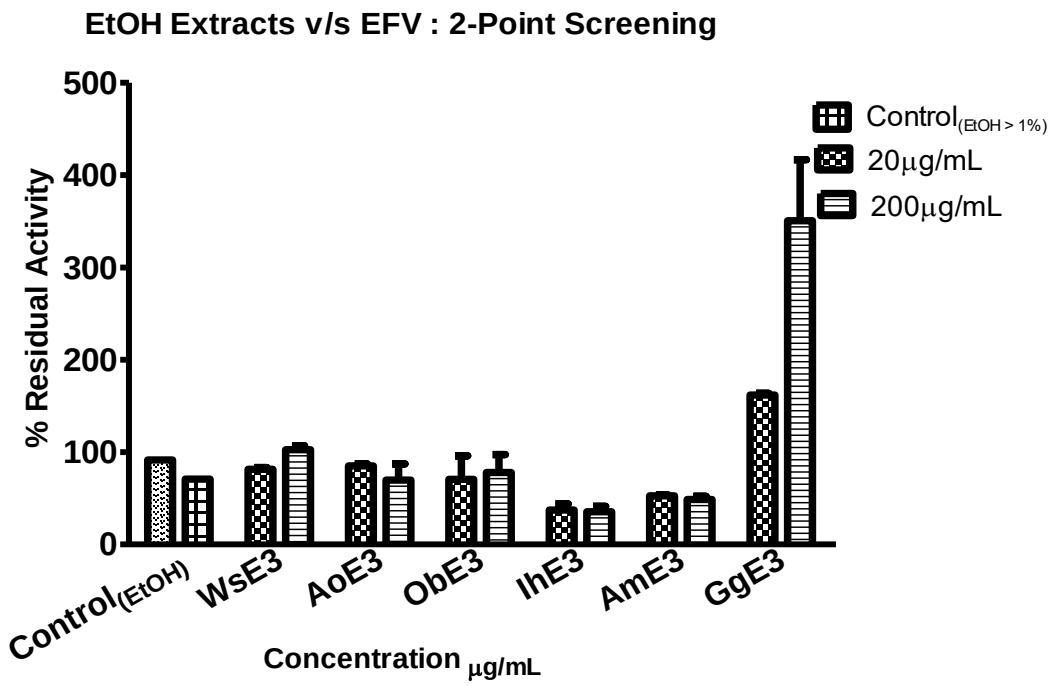


Fig.21 2-Point screening of all ethanolic extracts against CYP2B6 (Code name for each herb can be referred to table 2).

Amongst the ethyl acetate extracts, except for *W.somnifera* (WsEtA4) and *A.membranaceus* (AmEtA4), none of the extracts showed strong inhibition of CYP2B6 (Fig. 22). WsEtA4 reduced the activity to less than 60% and AmEtA4 reduced the activity to 47%. IhEtA4 inhibited CYP2B6 to a much lesser extent.

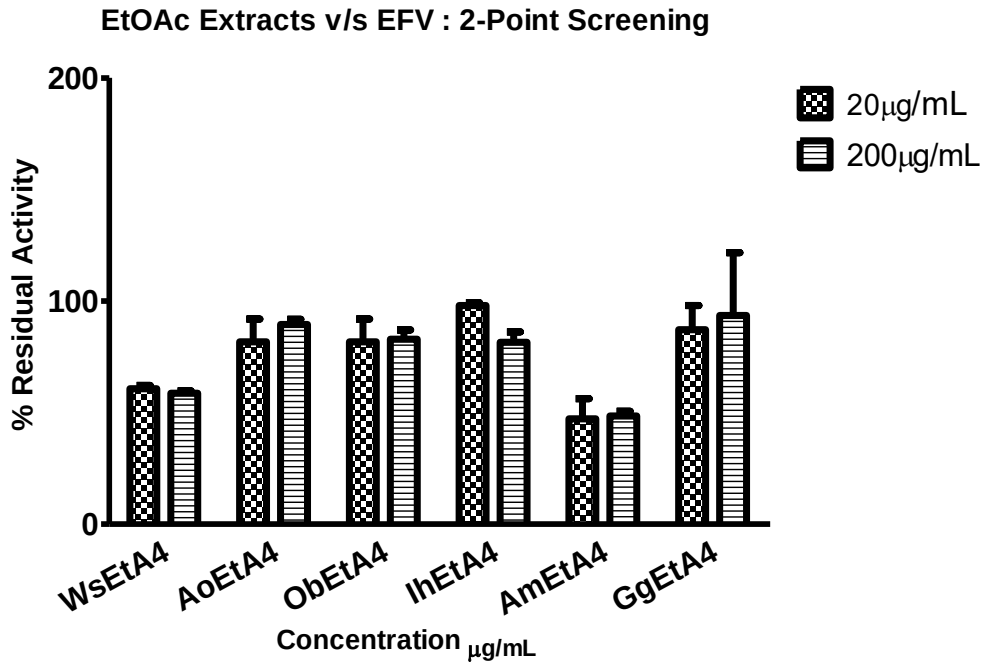


Fig.22 2-Point screening of all ethyl acetate extracts against RIF metabolism pathway (Code name for each herb can be referred to table 2).

Rifampicin metabolism: For the aqueous extracts screened against rifampicin metabolism in HLMs, most of the extracts increased formation of 25ODESRIF. Only IhA1 showed some inhibitory effect. ObA1 increased the percentage of remaining activity by almost 175%, while AmA1 induced by around 150% (Fig. 23).

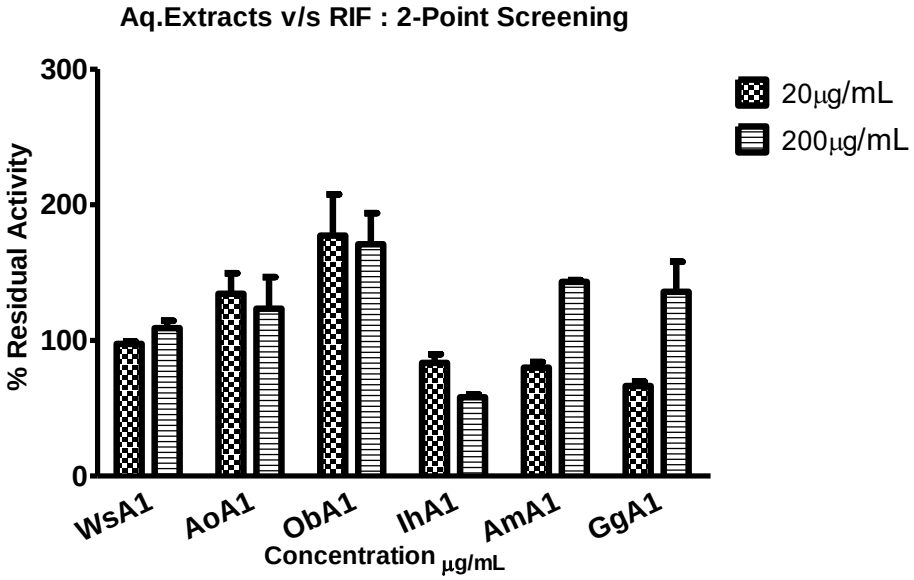


Fig.23 2-Point screening of all aqueous extracts against RIF metabolism pathway (Code name for each herb can be referred to table 2).

For the methanolic extracts, onlyObM2and IhM2 showed any inhibitory effect on the metabolism pathway, reducing the enzyme activity to less than 40% and 50% respectively. AmM2 extract showed increase in the formation of the metabolite similar to its aqueous extract. At a high concentration, the percentage activity increased by 150% for AmM2 (Fig. 24).

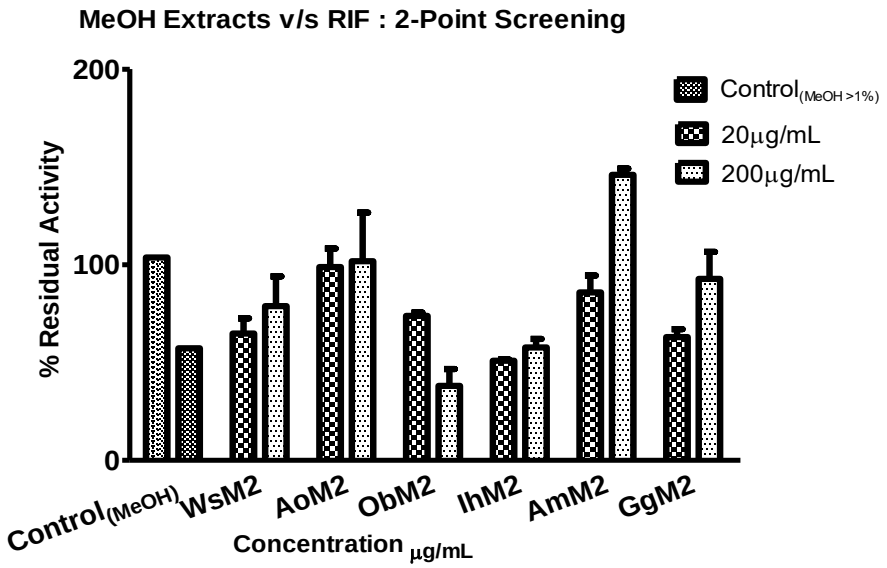


Fig.24 2-Point screening of all methanolic extracts against RIF metabolism pathway (Code name for each herb can be referred to table 2).

Similarly all ethanolic extracts enhanced the rate of formation of 25ODESRIF, except IhE3 andObE3 which reduced the activity to less than 40%. AmE3 showed the highest increase in the enzyme activity (Fig. 25).

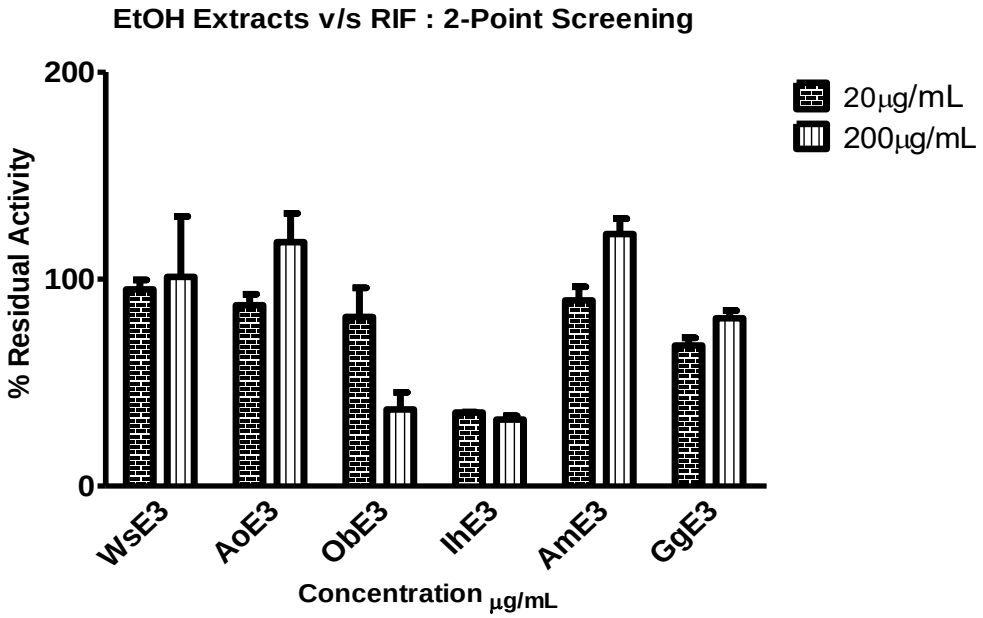


Fig.25 2-Point screening of all ethanolic extracts against RIF metabolism pathway (Code name for each herb can be referred to table 2).

For the ethyl acetate extracts (Fig. 26), only *I.helenium* (IhEtA4) reduced the metabolism of rifampicin to 35% at 200 μg/mL concentration. GgEtA4 showed modest increase in the metabolism rate at 200 μg/mL concentration.

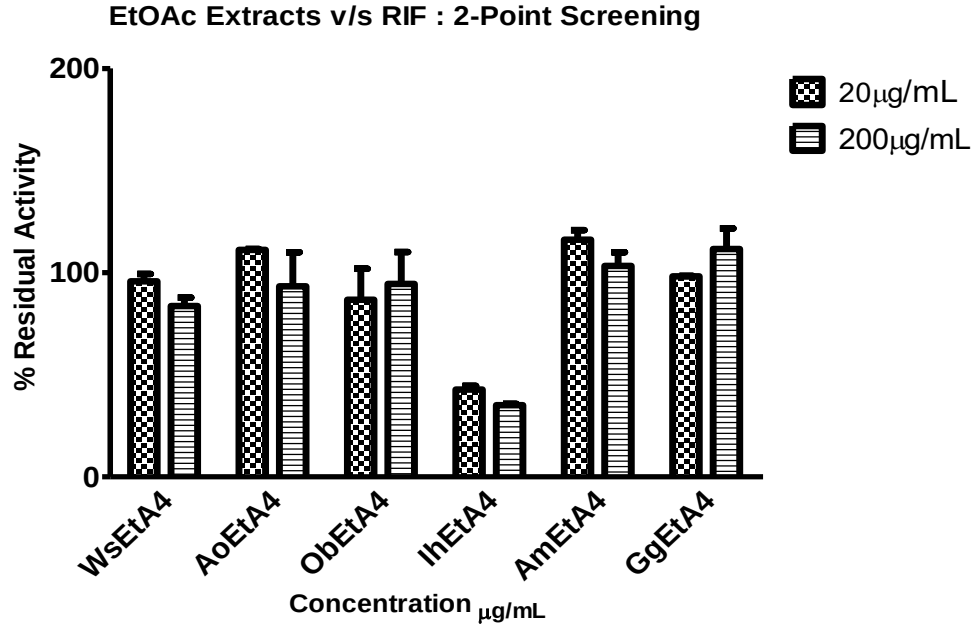


Fig.26 2-Point screening of all ethyl acetate extracts against RIF metabolism pathway (Code name for each herb can be referred to table 2).

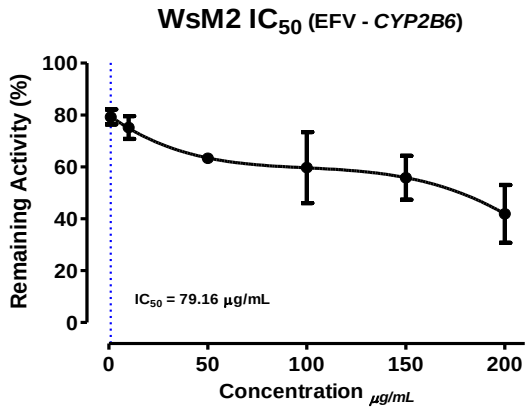
From all extracts screened, WsM2, WsEtA4, AmE3, AmEtA4, ObA1, ObM2, IhA1, IhM2 and IhE3 were selected as potential inhibitors of CYP2B6, and further screened for IC_{50} determination with TICL as the positive control. Extracts ObM2, ObE3, IhA1, IhM2, IhE3 and IhEtA4 were chosen as potential inhibitors of rifampicin metabolism for IC_{50} screening. All extracts of *Inula helenium* showed good inhibitory effect on both CYP2B6 and rifampicin metabolism.

3.3.4 IC_{50} determination of selected extracts

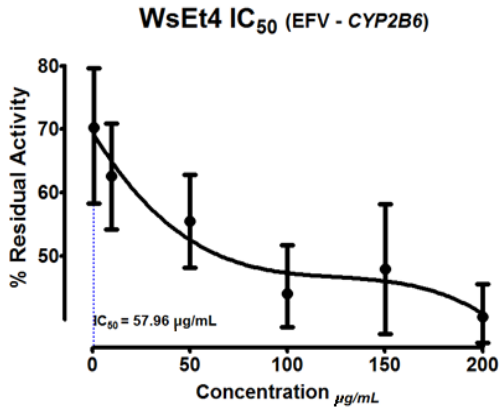
For CYP2B6 screening of the extracts, all IC_{50} values were in the range of 36-122 μg/mL for a concentration screening range between 1 and 200 μg/mL, which indicated that none of the extracts were potent inhibitors of CYP2B6 in that concentration range. ObM2 inhibited CYP2B6 with an IC_{50} value 36.07 μg/mL, while AmE3 had an IC_{50} value 53.37 μg/mL. IhA1 had an IC_{50} value 122.00 μg/mL. However at a higher concentration of 200 μg/mL the activity was less than 20% (Table 9).

Comparatively the positive control TICL had an IC_{50} value of 54.88 μM (14.47 μg/mL) when tested in a concentration range of 0-100 μM (Fig. 27) (Table 10).

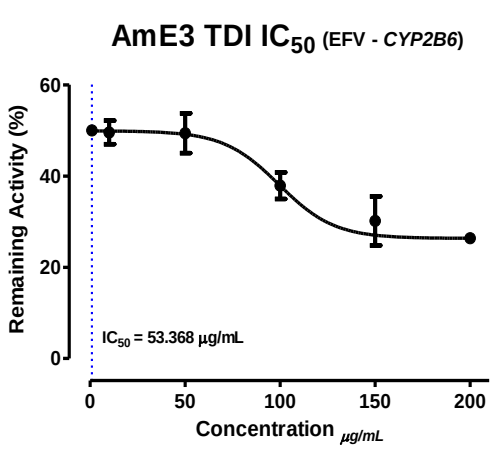
Both ethyl acetate extracts that were screened against CYP2B6 showed clear inhibition. For *Withania*, WsEtA4 showed more inhibition of CYP2B6 with an IC_{50} value 57.96 μg/mL compared to its methanolic extract (IC_{50} 79.16 μg/mL). Similar for *Astragalus*, the ethyl acetate extract inhibited CYP2B6 with an IC_{50} value 29.70 μg/mL and the ethanolic extract had an IC_{50} value 53.37 μg/mL.



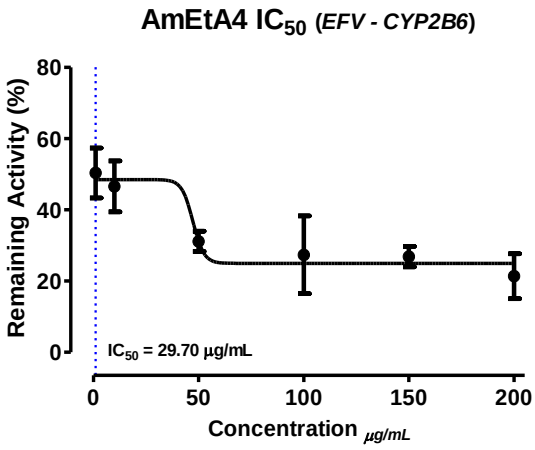
A



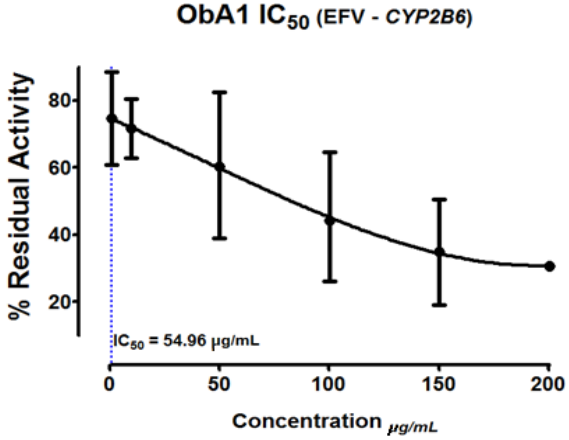
B


AmE3 TDI IC50 (EFV - CYP2B6)
Remaining Activity (%)
IC50 = 53.368 µg/mL
Concentration µg/mL

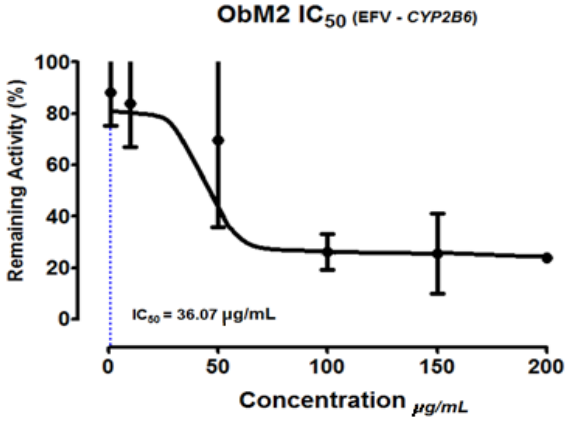
C


AmEtA4 IC50 (EFV - CYP2B6)
Remaining Activity (%)
IC50 = 29.70 µg/mL
Concentration µg/mL

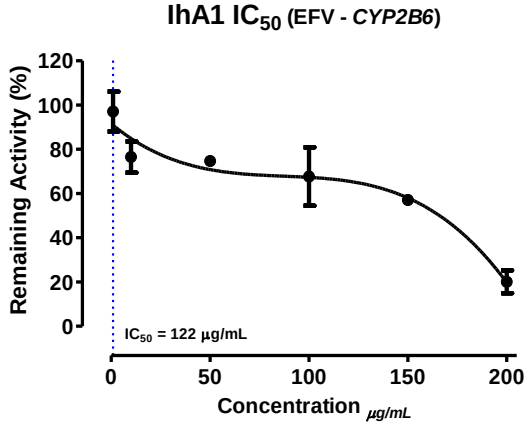
D


ObA1 IC50 (EFV - CYP2B6)
% Residual Activity
IC50 = 54.96 µg/mL
Concentration µg/mL

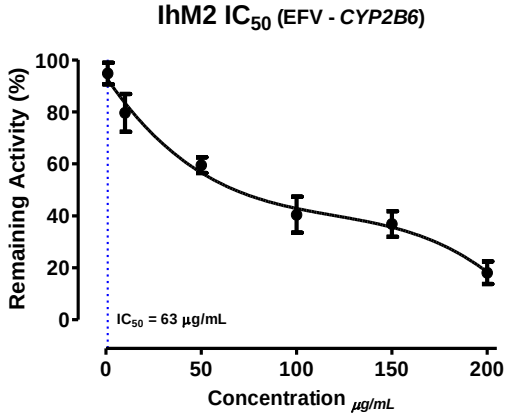
E


ObM2 IC50 (EFV - CYP2B6)
Remaining Activity (%)
IC50 = 36.07 µg/mL
Concentration µg/mL

F


IhA1 IC50 (EFV - CYP2B6)
Remaining Activity (%)
IC50 = 122 µg/mL
Concentration µg/mL

G


IhM2 IC50 (EFV - CYP2B6)
Remaining Activity (%)
IC50 = 63 µg/mL
Concentration µg/mL

H

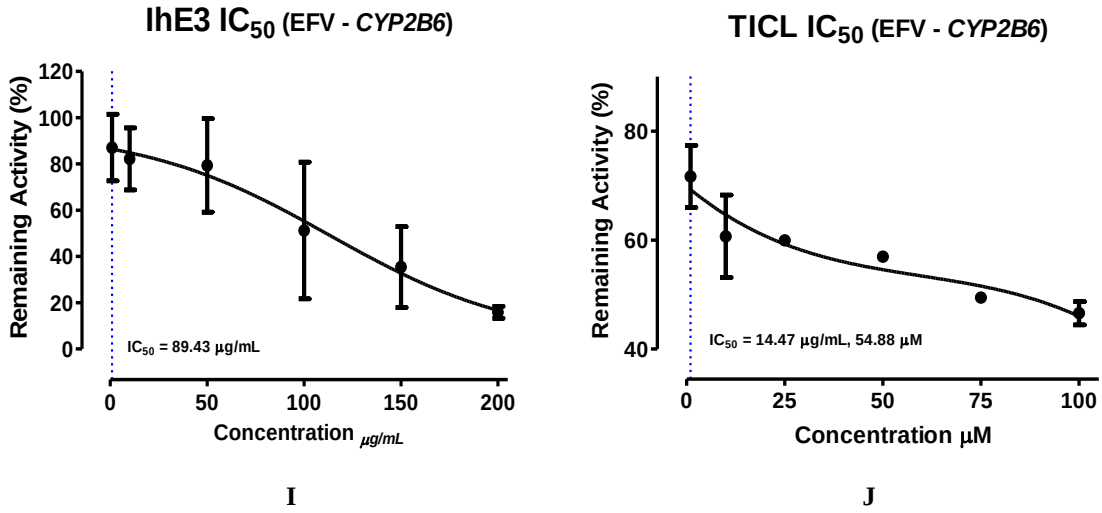
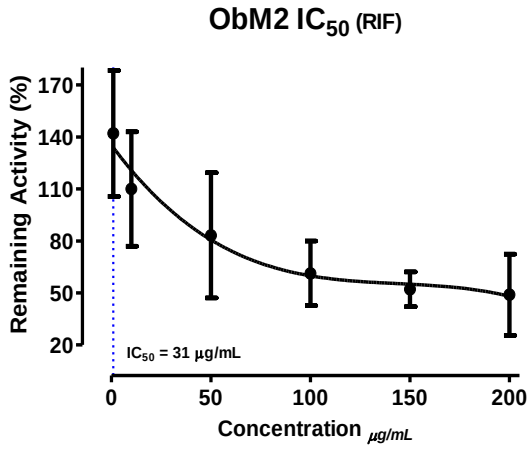


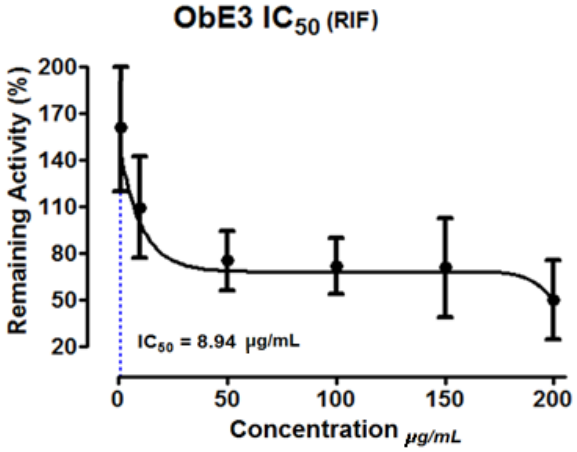
Fig.27 Statistical plots of herbal extract concentration (with IC_{50}s) of A: WsM2 (79.16 μg/mL), B: WsEtA4 (57.96 μg/mL), C: AmE3 (53.37 μg/mL), D: AmEtA4 (29.70 μg/mL), E: ObA1 (54.96 μg/mL), F: ObM2 (36.07 μg/mL), G: IhA1 (122 μg/mL), H: IhM2 (63 μg/mL), I: IhE3 (89.43 μg/mL) and positive control J: TICL (14.47 μg/mL, 54.88 μM) against percentage remaining activity of CYP2B6 with EFV as the substrate. The IC_{50} is calculated as log(X) against Y. The plot demonstrates the IC_{50} (μM) calculated using non-linear regression (dose-response inhibition) $^{v}/_{s}$ the actual IC_{50} plot curve-fit (Code name for each herb can be referred to table 2).

For the screening of extracts against rifampicin metabolism, all IC_{50} values were in the range of 8-108 μg/mL for a screening range between 1 and 200 μg/mL, indicating the potent inhibition effect of these extracts on the activity of the enzymes (Fig. 28) (Table 9). ObE3 inhibited the formation of 25ODESRIF, with the lowest IC_{50}value 8.94 μg/mL, while ObM2 had an IC_{50} value 31.00 μg/mL. IhE3 had an IC_{50} value 18.58 μg/mL (Fig. 28, E), while IhA1 and IhEtA4 inhibited rifampicin metabolism with values 108.00 μg/mL and 62.10 μg/mL, respectively (Fig. 28, C and F).

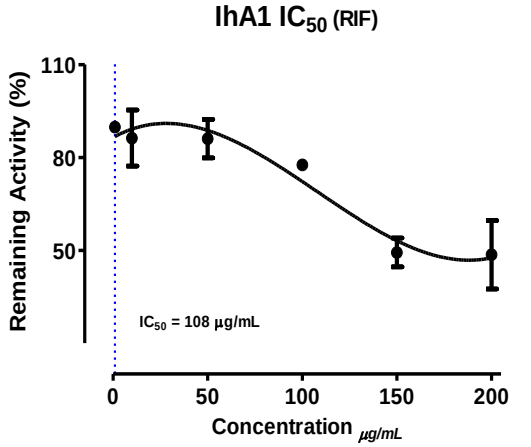
Comparatively it was observed that higher concentrations of NELF inhibited rifampicin metabolism in HLM and had an IC_{50} value of 9.59 μM (5.44 μg/mL) within a concentration range of 1-100 μM (Fig. 28, G) (Table 10).


ObM2 IC50 (RIF)
Remaining Activity (%)
170
140
110
80
50
20
IC50 = 31 µg/mL
0
50
100
150
200
Concentration µg/mL

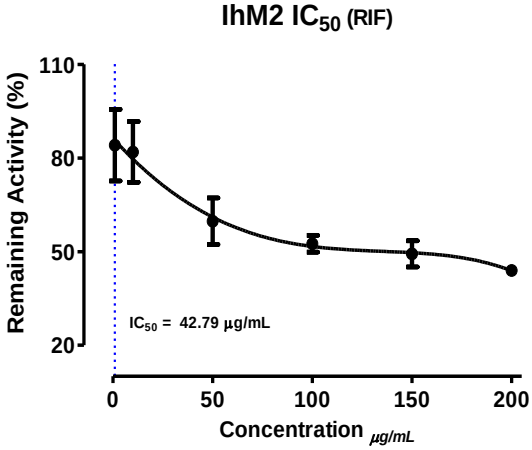
A


ObE3 IC50 (RIF)
Remaining Activity (%)
200
170
140
110
80
50
20
IC50 = 8.94 µg/mL
0
50
100
150
200
Concentration µg/mL

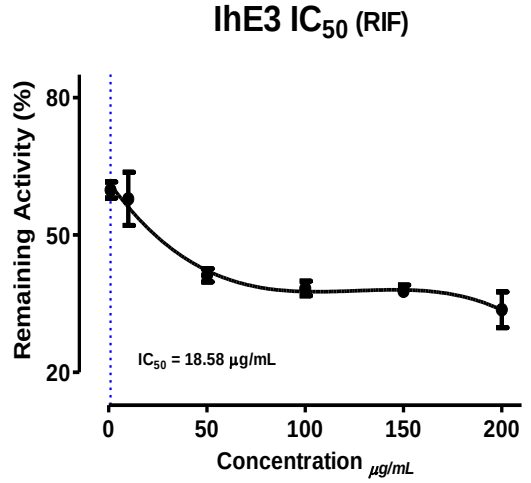
B


IhA1 IC50 (RIF)
Remaining Activity (%)
110
80
50
IC50 = 108 µg/mL
0
50
100
150
200
Concentration µg/mL

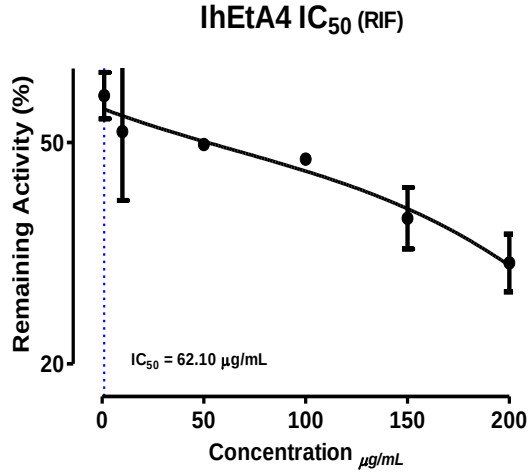
C


IhM2 IC50 (RIF)
Remaining Activity (%)
110
80
50
20
IC50 = 42.79 µg/mL
0
50
100
150
200
Concentration µg/mL

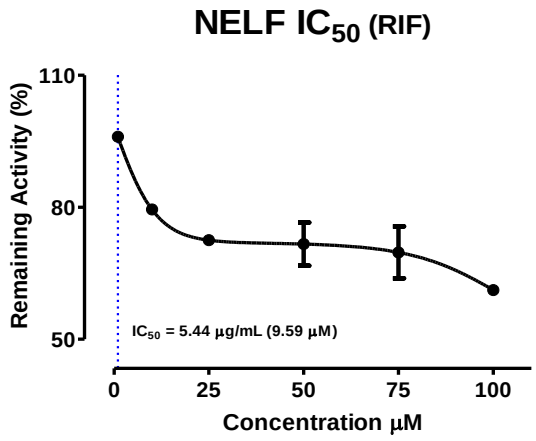
D


IhE3 IC50 (RIF)
Remaining Activity (%)
80
50
20
IC50 = 18.58 µg/mL
0
50
100
150
200
Concentration µg/mL

E


IhEtA4 IC50 (RIF)
Remaining Activity (%)
50
20
IC50 = 62.10 µg/mL
0
50
100
150
200
Concentration µg/mL

F



G

Fig.28 Statistical plots of herbal extract concentration (with IC_{50}s) of A: ObM2 (31.00 μg/mL), B: OBE3 (8.94 μg/mL), C: IhA1 (108.00 μg/mL), D: IhM2 (42.79 μg/mL), E: IhE3 (18.58 μg/mL), F: IhEtA4 (62.10 μg/mL) and positive control G: NELF (5.44 μg/mL, 9.59 μM) against percentage remaining activity of rifampicin metabolism. The IC_{50} is calculated as log(X) against Y. The plot demonstrates the IC_{50} (μM) calculated using non-linear regression (dose-response inhibition) $^{v}/_{s}$ the actual IC_{50} plot curve-fit (Code name for each herb can be referred to table 2).

Table [9] IC_{50} values of herbal extracts.

Extract	Extract code	CYP/Metabolism pathway	Determined IC_{50} values (μg/mL)
Withania somnifera	WsM1	CYP2B6	79.16
	WsEtA4	CYP2B6	57.96
Astragalus membranaceus	AmE3	CYP2B6	53.37
	AmEtA4	CYP2B6	29.70
Inula helenium	IhA1	CYP2B6	122.00
	IhM2	CYP2B6	63.00
	IhE3	CYP2B6	89.43
	IhA1	RIF metabolism	108.00
	IhM2	RIF metabolism	42.79
	IhE3	RIF metabolism	18.58
	IhEtA4	RIF metabolism	62.10
Ocimum basilicum	ObA1	CYP2B6	54.96
	ObM2	CYP2B6	36.07
	ObM2	RIF metabolism	31.00
	ObE3	RIF metabolism	8.94

Table [10] IC_{50} values of positive controls.

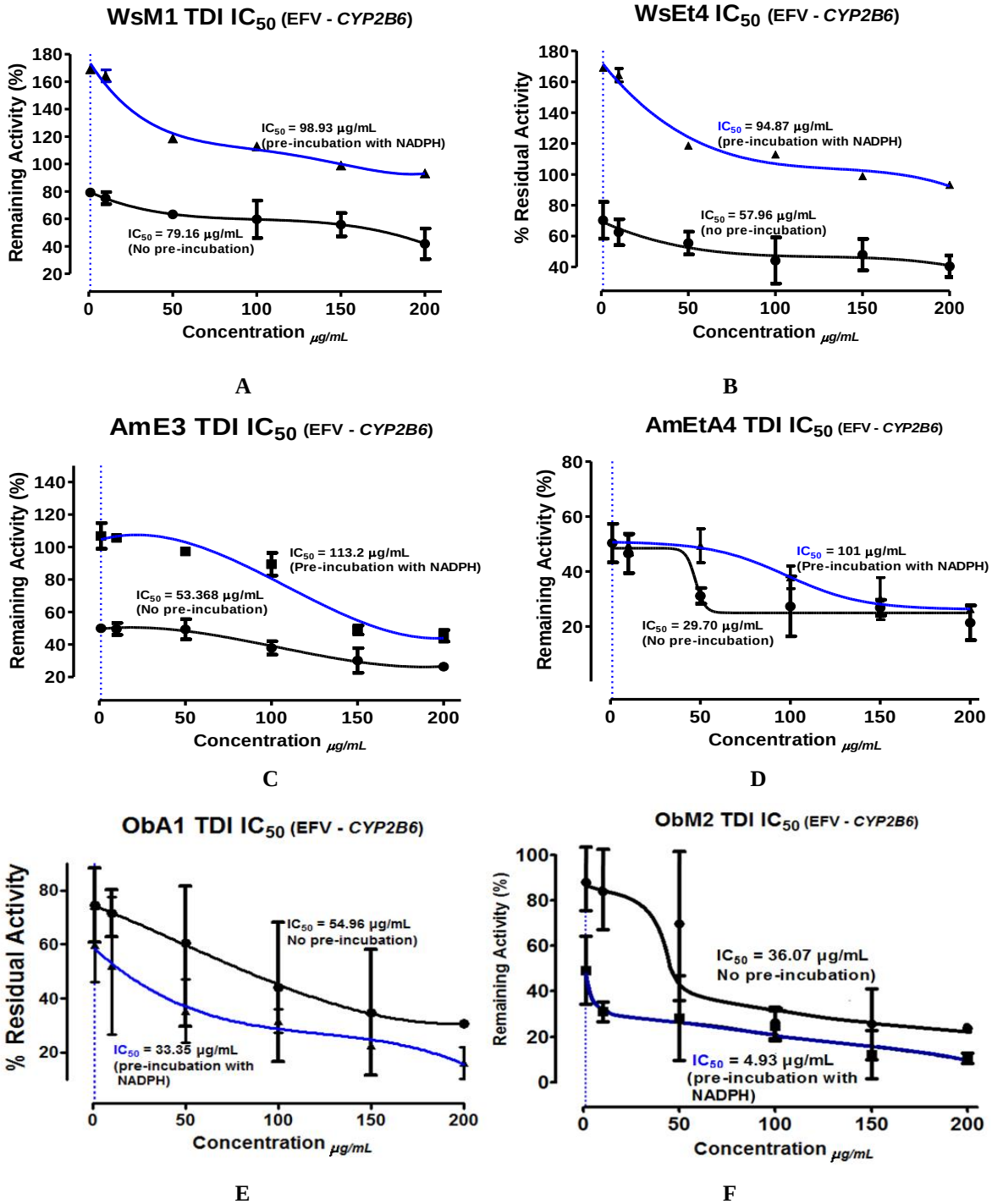
Positive Control	CYP/Metabolism pathway	Determined IC_{50} values (μg/mL)
Ticlopidine (TICL)	CYP2B6	14.47 (54.88 μM)
Nelfinavir (NELF)	RIF metabolism	5.44 (9.59 μM)

3.3.5 TDI IC_{50} fold shift determination of selected extracts

For TDI screening of the extracts, many of the extracts showed clear TDI of CYP2B6 activity (Fig. 29a, b). The aqueous, methanolic and ethyl acetate extracts of *Inula helenium* showed

clear TDI of CYP2B6 with values 40.37, 33.00 and 66.48 μg/mL when compared to their normal IC_{50}s which did not involve preincubation with NADPH (Table. 11). Both the *Ocimum basilicum* extracts also showed TDI of CYP2B6 and ObM2 had a shift of IC_{50} from 36.07 μg/mL to 4.93 μg/mL. The ethyl acetate extracts WsEtA4 and AmEtA4 showed no TDI. Compared to the IC_{50} for the assays with no pre-incubation of NADPH, both extracts had higher TDI IC_{50} values of 94.87 μg/mL and 101.00 μg/mL, respectively (Fig. 29a, B & D).

The positive control TICL showed TDI where the IC_{50} shifted to 36.51 μM (9.63 μg/mL) (Fig. 29a, J).



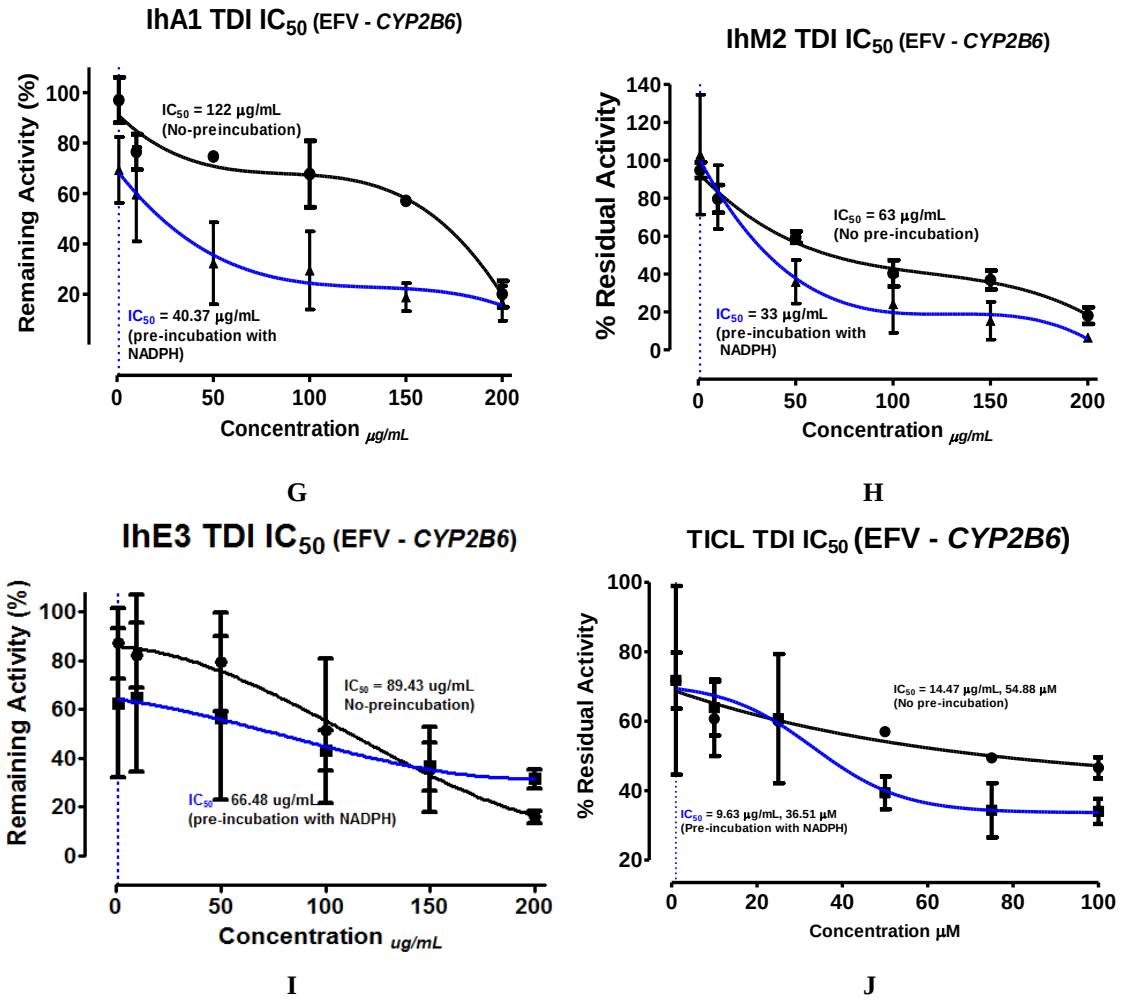


Fig.29a Statistical TDI plots of herbal extract concentration (with TDI IC_{50}s) of A: WsM2 (98.93 μg/mL), B: WsEtA4 (94.87 μg/mL), C: AmE3 (113.20 μg/mL), D: AmEtA4 (101.00 μg/mL), E: ObA1 (33.35 μg/mL), F: ObM2 (4.93 μg/mL), G: IhA1 (40.37 μg/mL), H: IhM2 (33.00 μg/mL), I: IhE3 (66.48 μg/mL) and positive control J: TICL (9.63 μg/mL, 36.51 μM) against percentage remaining activity of CYP2B6 with EFV as the substrate. The IC_{50} is calculated as log(X) against Y. The plot demonstrates the IC_{50} (μM) calculated using non-linear regression (dose-response inhibition) $^{v}/_{s}$ the actual IC_{50} plot curve-fit.

For the TDI screening of the extracts against rifampicin metabolism pathway, none of the extracts showed a trend for inhibiting the activity, on preincubation with NADPH, except for IhM2 where the IC_{50} shifted from 42.79 to 23.11 μg/mL (Table. 11). Some of the extracts even had an IC_{50} shift of > 100 μg/mL on preincubation with NADPH (Fig. 29b).

The ethyl acetate extract IhEtA4, showed an IC_{50} value 62.10 μg/mL in the assay without pre-incubation of NADPH. For the TDI assay, the IC_{50} shift observed for the extract was > 100 μg/mL (Fig. 29b, F).

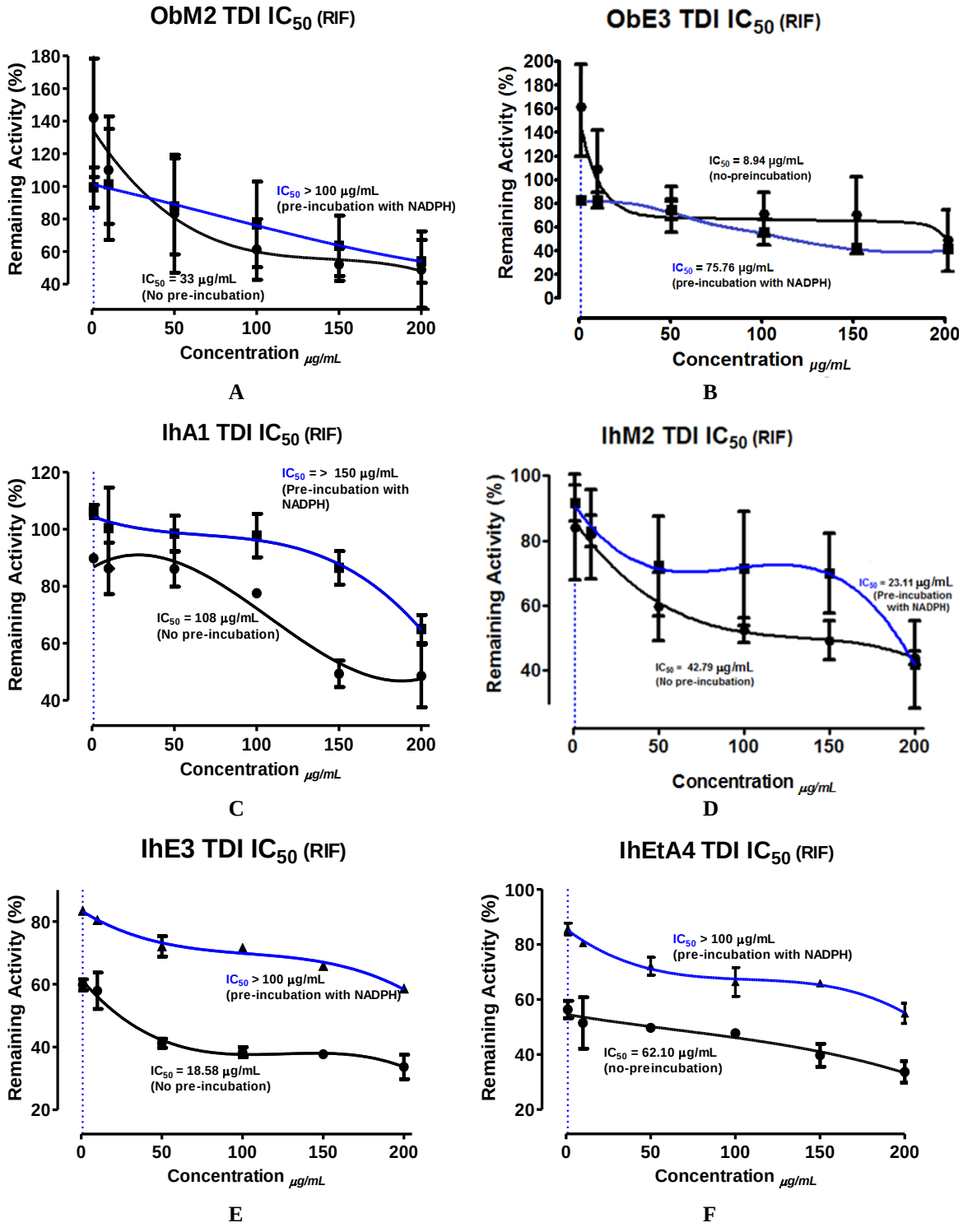


Fig.29b Statistical TDI plots of herbal extract concentration (with TDI IC_{50}s) of A: ObM2 (>100 μg/mL), B: OBE3 (75.76 μg/mL), C: IhA1 (>150 μg/mL), D: IhM2 (23.11 μg/mL), E: IhE3 (>100 μg/mL), F: IhEtA4 (62.10 μg/mL) against percentage remaining activity of rifampicin metabolism. The IC_{50} is calculated as log(X) against Y. The plot demonstrates the IC_{50} (μM) calculated using non-linear regression (dose-response inhibition) $^{v}/_{s}$ the actual IC_{50} plot curve-fit.

NELF showed clear TDI of the metabolism, where the IC_{50} value shifted from 9.59 μM (5.44 μg/mL) to 6.39 μM (3.63 μg/mL) (fig 29c).

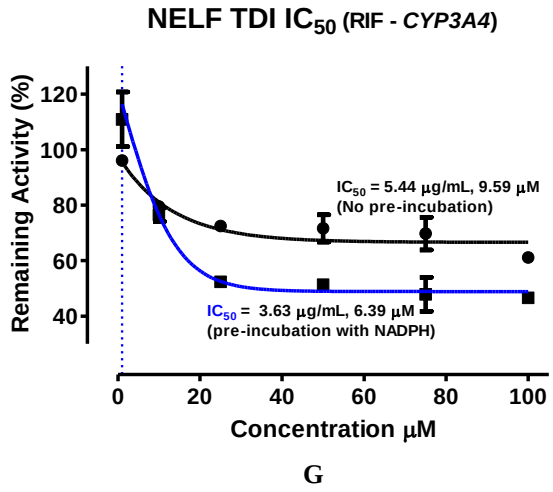


Fig.29c Statistical TDI plot of the positive control G: NELF (TDI IC_{50}= 3.63 μg/mL, 6.39 μM) against percentage remaining activity of rifampicin metabolism. The IC_{50} is calculated as log(X) against Y. The plot demonstrates the IC_{50} (μM) calculated using non-linear regression (dose-response inhibition) $^{v}/_{s}$ the actual IC_{50} plot curve-fit.

Table [11] TDI IC_{50}Shift-fold values of herbal extracts and positive controls.

Extract	Extract code	CYP/ Metabolism pathway	Determined IC_{50} values IC_{50}(-)(μg/mL)	TDI IC_{50} values IC_{50}(+) (μg/mL)	TDI fold-shift IC_{50}(-)/IC_{50}(+)
Withania somnifera	WsM1	CYP2B6	79.16	98.93	<1.5
	WsEtA4	CYP2B6	57.96	94.87	< 1.5
Astragalus membranaceus	AmE3	CYP2B6	53.37	113.20	< 1.5
	AmEtA4	CYP2B6	29.70	101.00	< 1.5
Inula helenium	IhA1	CYP2B6	122.00	40.37	> 1.5
	IhM2	CYP2B6	63.00	33.00	> 1.5
	IhE3	CYP2B6	89.43	66.48	<1.5
	IhA1	RIF metabolism	108.00	> 150	<1.5
	IhM2	RIF metabolism	42.79	23.11	>1.5
	IhE3	RIF metabolism	18.58	> 100	<1.5
	IhEtA4	RIF metabolism	62.10	> 100	<1.5
Ocimum basilicum	ObA1	CYP2B6	54.96	33.35	>1.5
	ObM2	CYP2B6	36.07	4.93	>1.5
	ObM2	RIF metabolism	31.00	> 100	<1.5

	ObE3	**RIF metabolism**	**8.94**	**75.76**	**<1.5**
Ticlopidine	**TICL**	**CYP2B6**	**14.47 (54.88 μM)**	**9.63 (36.51 μM)**	**>1.5**
Nelfinavir	**NELF**	**RIF metabolism**	**5.44 (9.59 μM)**	**3.63 (6.39 μM)**	**>1.5**

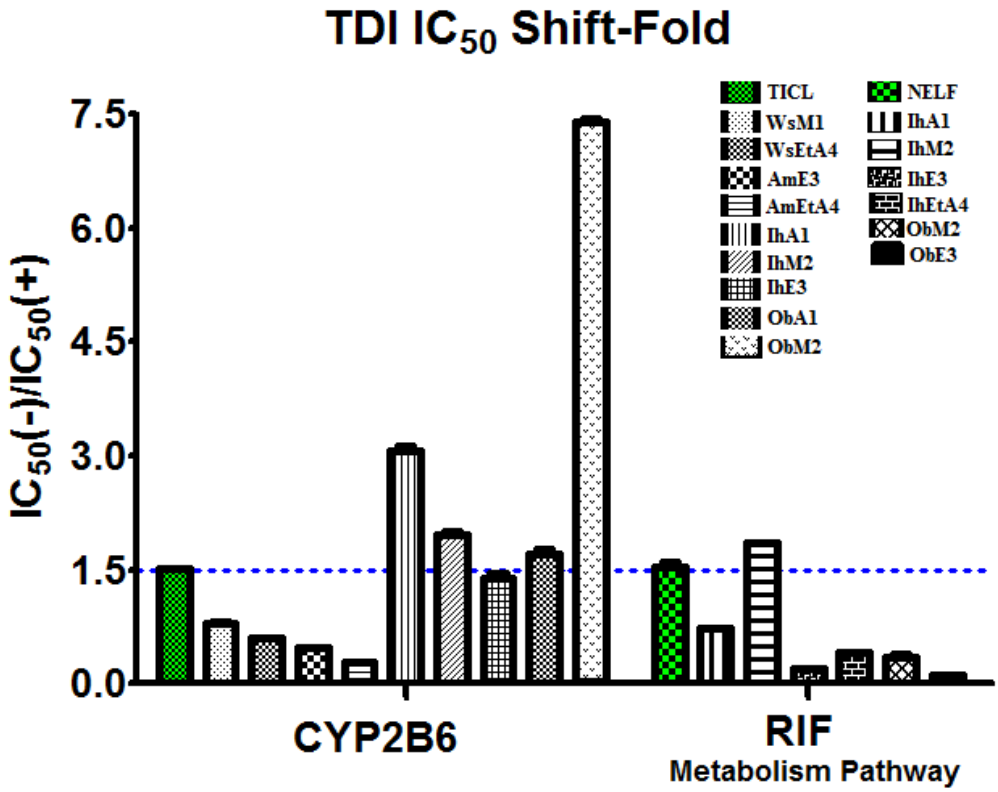


Fig.30 Determination of TDI based on IC_{50} shift-fold. Bars represent the ratio of IC_{50} values for the co-incubation(-) to the preincubation (+) assays with NADPH, for the herbal extracts. TICL and NELF were positive controls for CYP2B6 and RIF metabolism pathway, respectively.

For the TDI determination, the IC_{50} shift-fold was calculated as the ratio of the co-incubation IC_{50}(-) to the pre-incubation IC_{50}(+) with NADPH (Fig. 30). IhA1, IhM2, ObA1 and ObM2 showed positive TDI for CYP2B6. IhA1 showed 3-fold increase in the IC_{50} against CYP2B6 and IhM2 had 2-fold shift, for rifampicin metabolism.ObM2 exhibited strong TDI on CYP2B6 with 7.4-fold increase in the IC_{50}.

Both positive controls TICL and NELF showed clear TDI with the IC_{50} shift-fold >1.5.

3.4 Phytochemical fingerprinting – Extracts inhibiting CYP activity

3.4.1 LC-MS/ PDA analysis

Gallic acid and quercetin were used as reference standards for relative quantification of the identified acid compounds and non-acid compounds, respectively. Majority of the acid

compounds were detected in negative scan mode and hence, gallic acid calibration was determined in negative scan mode (table 12, fig. 31).

The linearity characteristics of the LC-MS method for both standards are shown in Table 8.

Table [12] LC-MS analysis characteristics.

Reference Standard	Quercetin		Gallic Acid	
Retention time (min)	**24.21**		**6.73**	
$[M-H]^-$	**169.1001**		**301.1211**	
Linearity range (mg/L)	**200.000 – 6.250**		**200.000 – 6.250**	
Linear Correlation Coefficient (R^2)	**$-^{ve}$ scan mode**	**0.9442**	**$-^{ve}$ scan mode**	**0.9700**
	$+^{ve}$ scan mode	**0.9900**		
UV range	**Between 230 and 600 nm**			

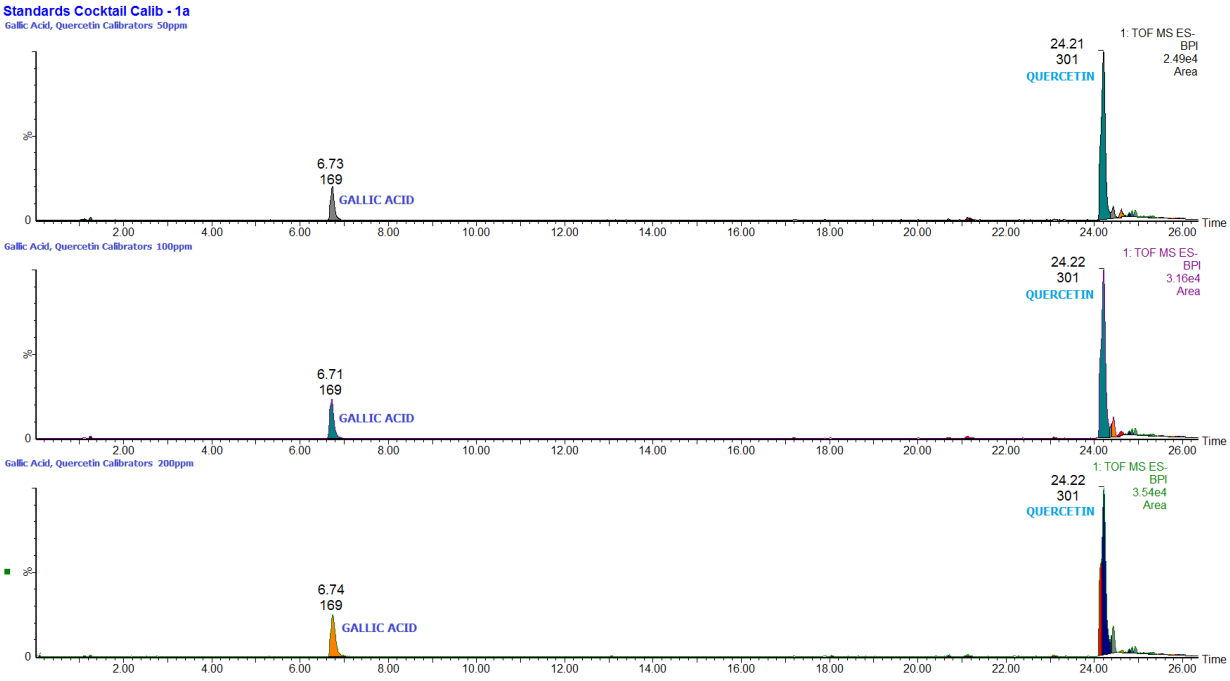


Fig.31 Gallic acid and quercetin scans on LC-MS at 200.000, 100.000 and 50.000 mg/L injections.

All negative scans were performed for 29 min, while positive scans were performed for 15 min. The major peaks and their masses were used in the tentative identification of the phytocompounds based on database search, *m/z* transitions and the retention time match from literature.

For the negative mode extract scans, the following main findings were observed (for details, see table 13):

1. *Ocimum basilicum* (aqueous, methanol and ethanol) extracts (fig. 32 shows the chromatogram of the aqueous extract):
    - The most prominent peak in all extracts of *O.basilicum* was the polyphenol - rosmarinic acid (fig. 33 a) with a retention time of 21.65 min at *m/z* 359 at a PDA detection wavelength 329nm.
    - Another prominent peak observed in all three extracts was the flavone salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone), at retention time 24.36 min at *m/z* 327 with product ions at *m/z* 116.9, 205, 215, 277 and 311 (fig. 33 b).
    - Acids such as tartaric, isocitric, caftaric and chicoric acids were prominently observed in the aqueous extract at *m/z* 149, 191, 311 and 473, at UV wavelengths 230 nm for the first two and 328-329 nm for the latter two compounds (fig. 33 c, d, e).
    - The flavanoid rutin was another major peak observed only in the methanolic extract at *m/z* 609 with products ions at *m/z* 151, 255, 271, 300 and 301 (fig. 33 f).
    - Apigenin-7-O-glucoside was observed in the ethanolic extract with a retention time 14.76 min and *m/z* 431.
    - Other compounds detected in the extracts included 12-hydroxyjasmonic acid (225 *m/z*), medioresinol (387 *m/z*), trans-Ocimene oxide (137 *m/z*) and eucommiol (187 *m/z*).

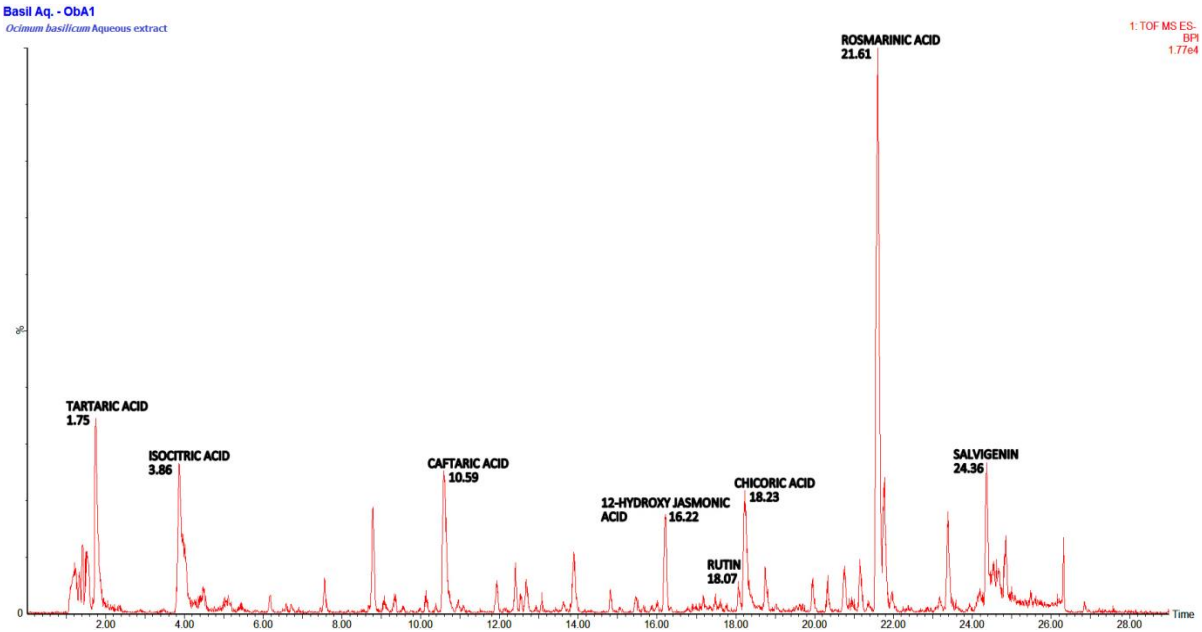


Fig. 32 LC-MS chromatogram of *O.basilicum* (aq. extract) in negative mode scan for 29 min.

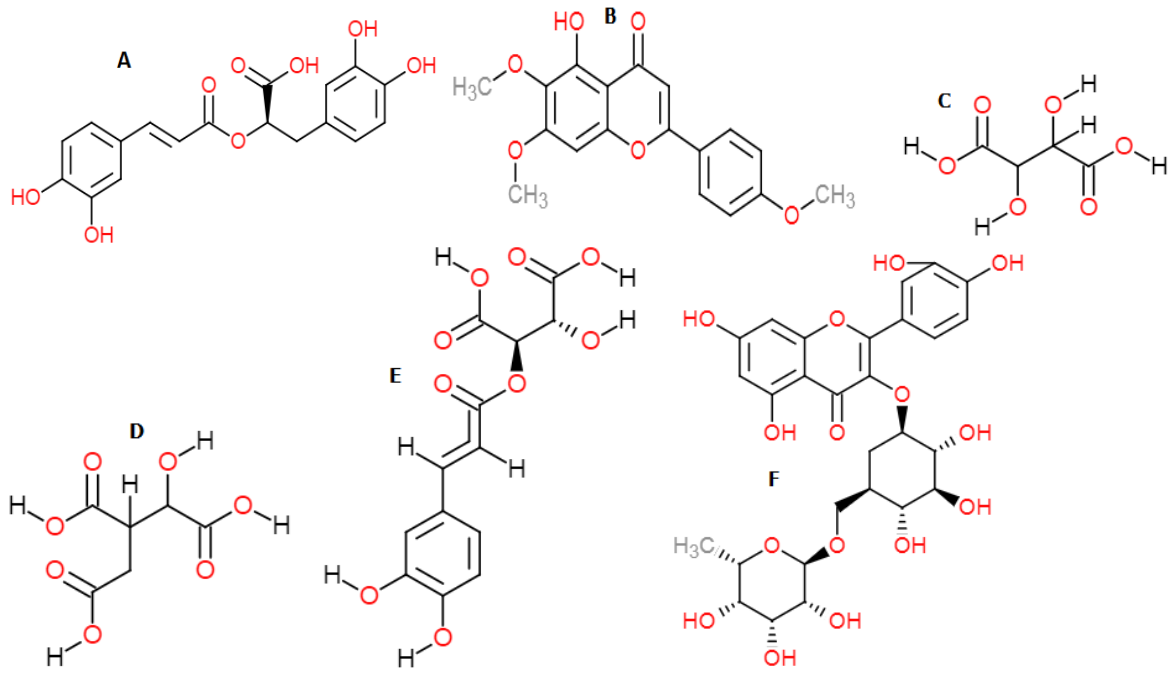


Fig. 33 Structures of some of the main compounds identified from crude herbal extracts of *O.basilicum*: (A) Rosmarinic acid; (B) Salvigenin; (C) Tartaric acid; (D) Isocitric acid; (E) Caftaric acid; (F) Rutin

2. *Inula helenium* (aqueous, methanol, ethanol and ethyl acetate) extracts (fig. 34 shows the ethanol extract chromatogram):
   - The most prominent peak in all organic solvent extracts of *I.helenium* was identified as the sesquiterpenoid tanacetol A (2-acetoxy-11-hydroxy-1(10),4(15)-germacradien-5-one) (fig.35 a) with a retention time of 24.77 min at *m/z* 293 at a UV detection wavelength 275 nm, and product ions at *m/z* 87, 116 and 231.
   - Macrophyllilactone B, which was previously reported in *I.helenium* extracts, was observed in all three organic solvent extracts at retention time 26.3 min at *m/z* 265 with product ions at *m/z* 112.98, 183 and 248.95 (fig. 35 b).
   - The aqueous extract had a major peak identified as a dihexose sugar, at 1.79 min and *m/z* 341.
   - Three quinic acid derivatives – chlorogenic acid, cryptochlorogenic acid and 5-caffeoylquinic acid were observed in the aqueous extract (fig. 35 c, d, e) at *m/z* 353 and distinguished based on the product ions as in table 11.
   - Compounds such as isocitric acid and fukugetin were identified in the aqueous extract at *m/z* 191 and 555, respectively.

- The polyphenol epigallocatechin gallate was present in all the extracts but most prominent in the methanolic extract at *m/z* 457 and product ions at *m/z* 112, 191, 209 and 371, at retention time 19.57 min.
- Chlorogenic acid derivative (quinic acid isomer) was observed in the methanol and ethanol extracts at *m/z* 191, retention 12.71 min and daughter ions *m/z* 85, 135, 161, 179 (Zhang et al., 2016), while the known compound 5alpha-epoxyalantolactone was noticed only in the aqueous extract at *m/z* 247 and product ions *m/z* 115.92, 197, 221 and 233.

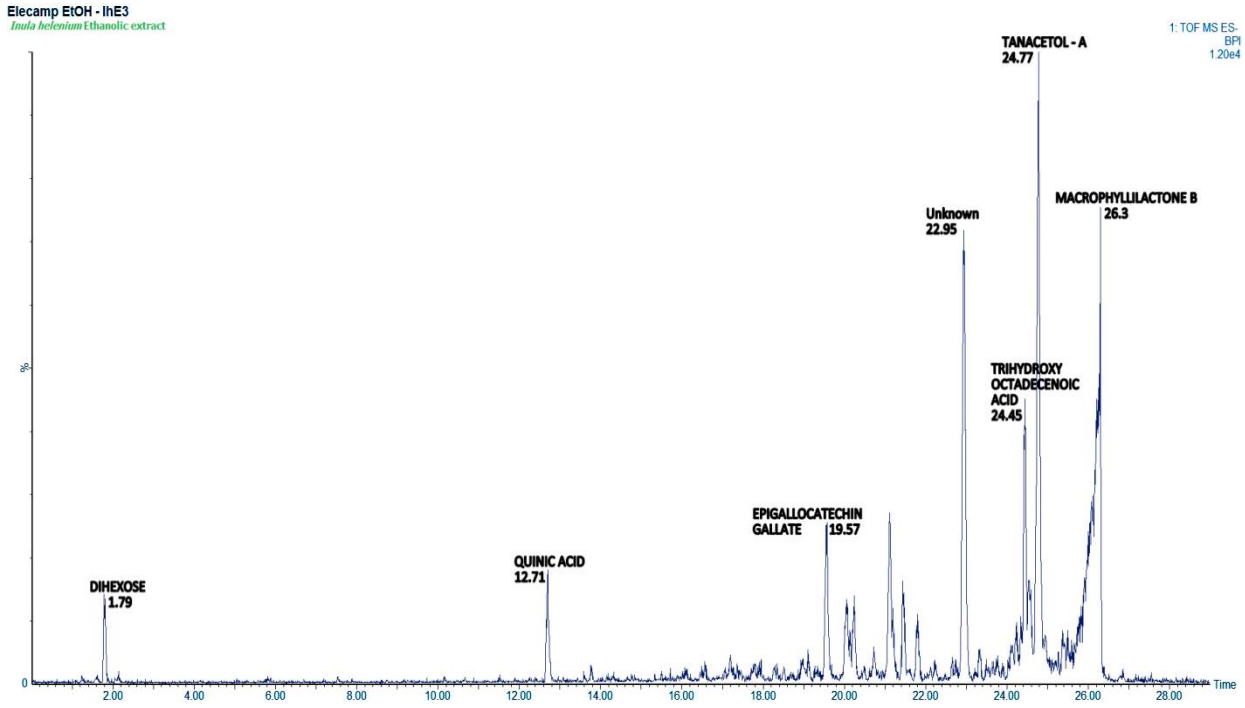
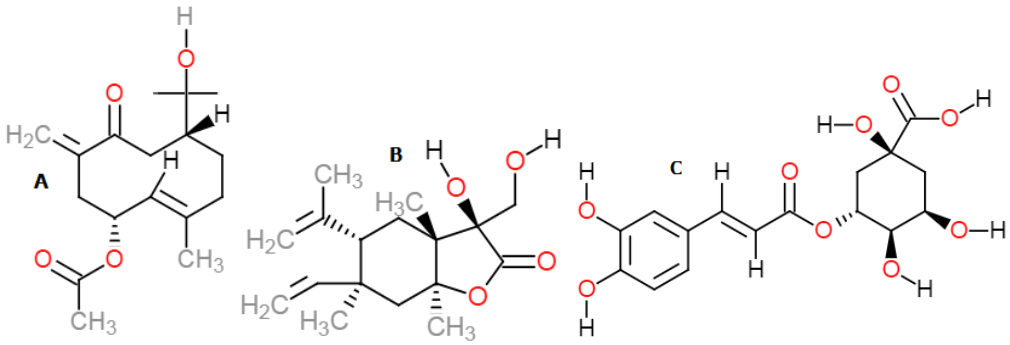


Fig. 34 LC-MS chromatogram of *I.helenium* (ethanol extract) in negative mode scan for 29 min.



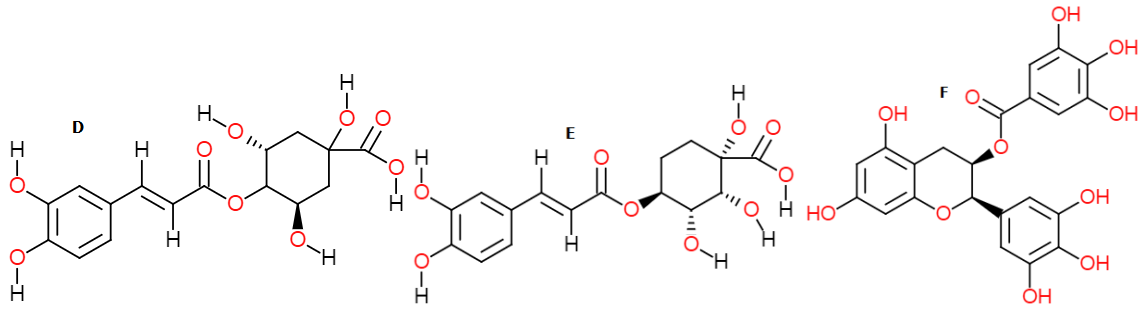


Fig. 35 Structures of some of the main compounds identified from crude herbal extracts of *I.helenium*: (A) Tanacetol A; (B) Macrophyllilactone B; (C) Chlorogenic acid (D) Cryptochlorogenic acid; (E) 5-caffeoylquinic acid and; (F) Epigallocatechin gallate

3. *Withania somnifera* (methanol and ethyl acetate) extracts (fig. 36 shows the chromatogram of the ethyl acetate extract):
    - The most prominent peaks in the methanol extract of *W.somnifera* were observed at retention times 24.36 and 24.12 min at *m/z* 811 and 827 respectively, at a UV detection wavelength 230 nm. The product ions for the main peak were *m/z* 179, 329, 503, 765 and the same for the second major peak were *m/z* 179, 312, 503, 653 and 781. These compounds did not match any of the previously identified compounds, but the molecular formula and the corresponding masses indicated that they could probably be withanoside derivatives or withanolide complexes.

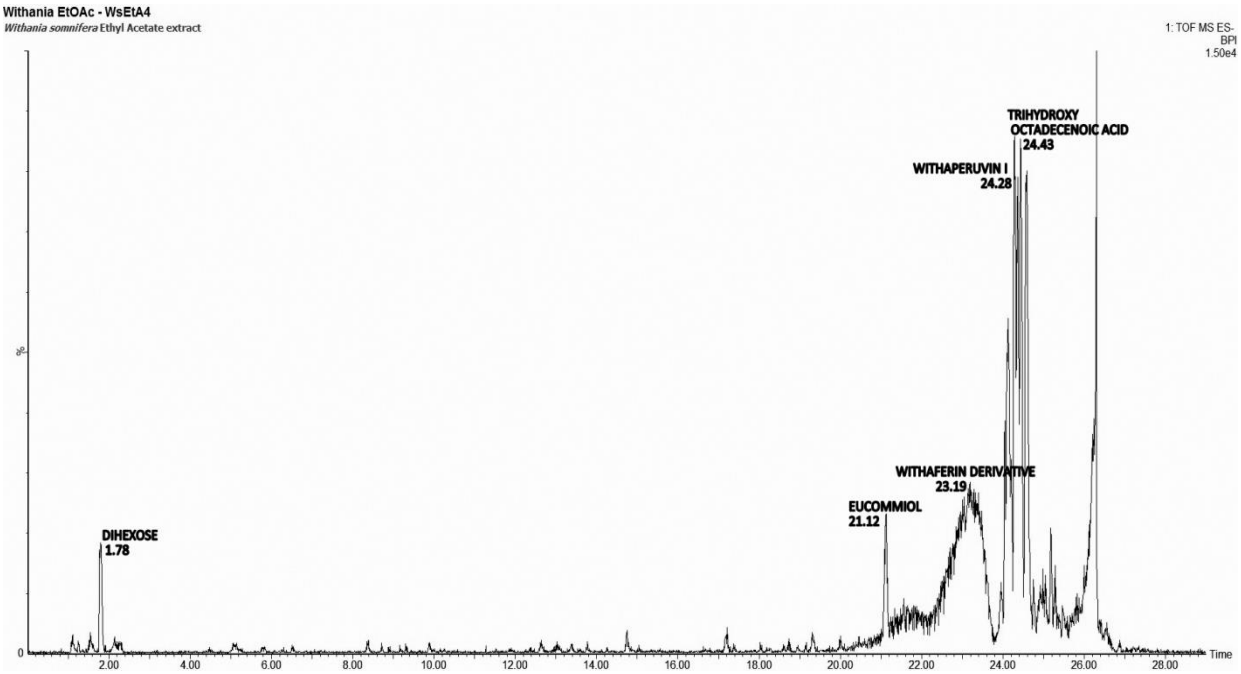


Fig. 36 LC-MS chromatogram of *W.somnifera* (ethyl acetate extract) in negative mode scan for 29 min.

- Salvigenin was observed in the methanol extract (fig. 37 b) at 24.57 min, while the alkaloid isopelletierine (1-pyridin-2-ylpropan-2-one) was observed at 2.11 min (fig. 37 c) with *m/z* 133 and product ions *m/z* 43, 71 and 93.
- The known compounds withaperuvin I and withaferin derivative, were observed in the ethyl acetate extract at 230 nm, with retention times 24.28 and 23.10 min, respectively, while the steroidal lactone dihydrowithaferin A (472 *m/z*) was observed in the aqueous extract at 13.09 min at 280 nm (fig. 37 d).
- Other peaks identified included the fatty acid trihydroxy octadecenoic acid (*m/z* 329) and eucommiol (187 *m/z*) at retention times 24.43 and 21.12 min (fig. 37 e) respectively.

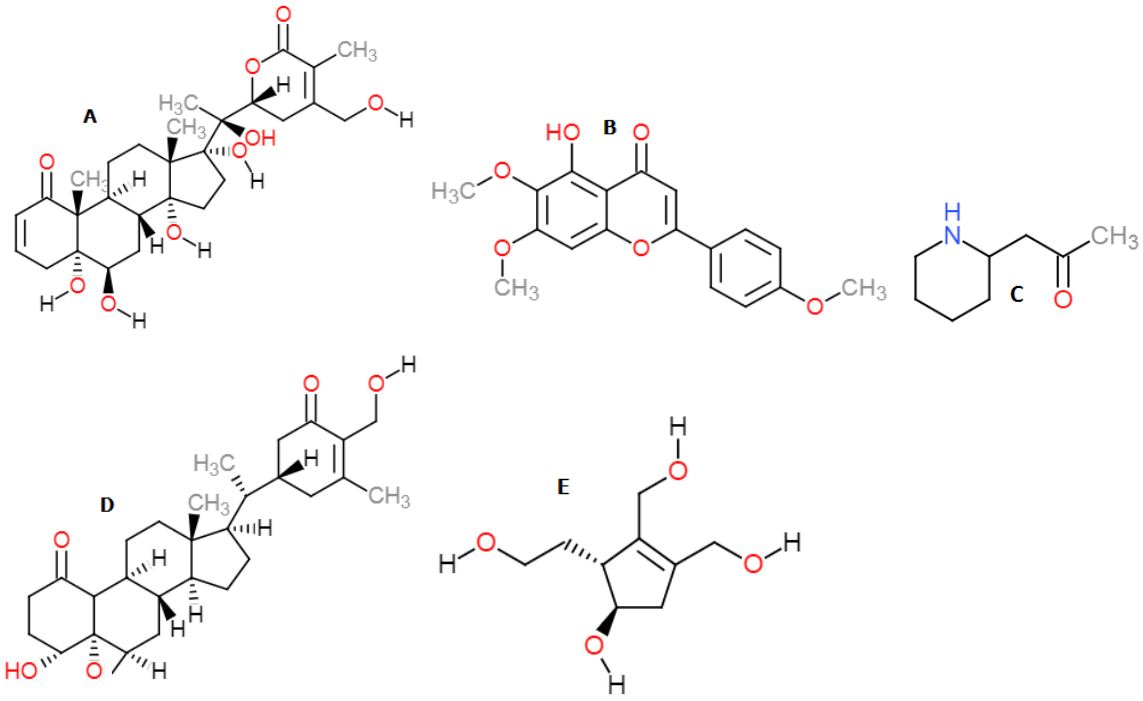


Fig. 37 Structures of some of the main compounds identified from crude herbal extracts of *W.somnifera*: (A) Withaperuvin I; (B) Salvigenin; (C) Isopelletierine; (D) Dihydrowithaferin A; (E) Eucommiol.

4. *Astragalus membranaceus* (methanol, ethanol and ethyl acetate) extracts (fig. 38 illustrates the chromatogram of the methanol extract):
   - Prominent peaks in all the extracts of *A.membranaceus* were identified as the isoflavonoids calycosin (7,3'-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone) and observed at retention times 24.22 and 24.57 min with *m/z* 283 and 267, respectively, at a UV detection wavelength 230 nm (fig. 39 a, b). The product ions for calycosin were *m/z* 136, 196, 211, 240, 269 and the same for formononetin were *m/z* 115, 117, 196, 207 and 236.

- A dihexose sugar (341 *m/z*) was observed in the methanol and ethanol extracts at 1.79 min, with product ions identified at *m/z* 89, 101, 119, 161 and 179.
- The fatty acid trihydroxy octadecenoic acid (329 *m/z*) was observed at 24.43 min in all extracts, with product ions *m/z* 116.92, 171, 211and 258 (fig. 39 d).
- Other minor peaks included the compounds trifolirhizin (491 *m/z*) and eucommiol (187 *m/z*).

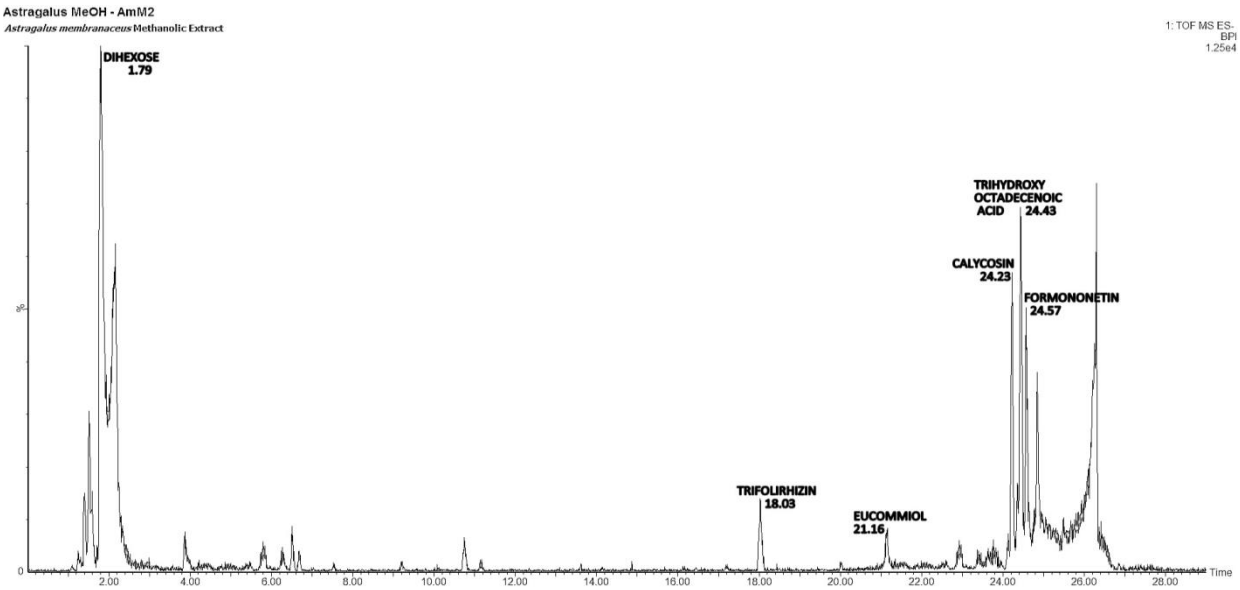


Fig. 38 LC-MS chromatogram of *A.membranaceus* (methanol extract) in negative mode scan for 29 min.

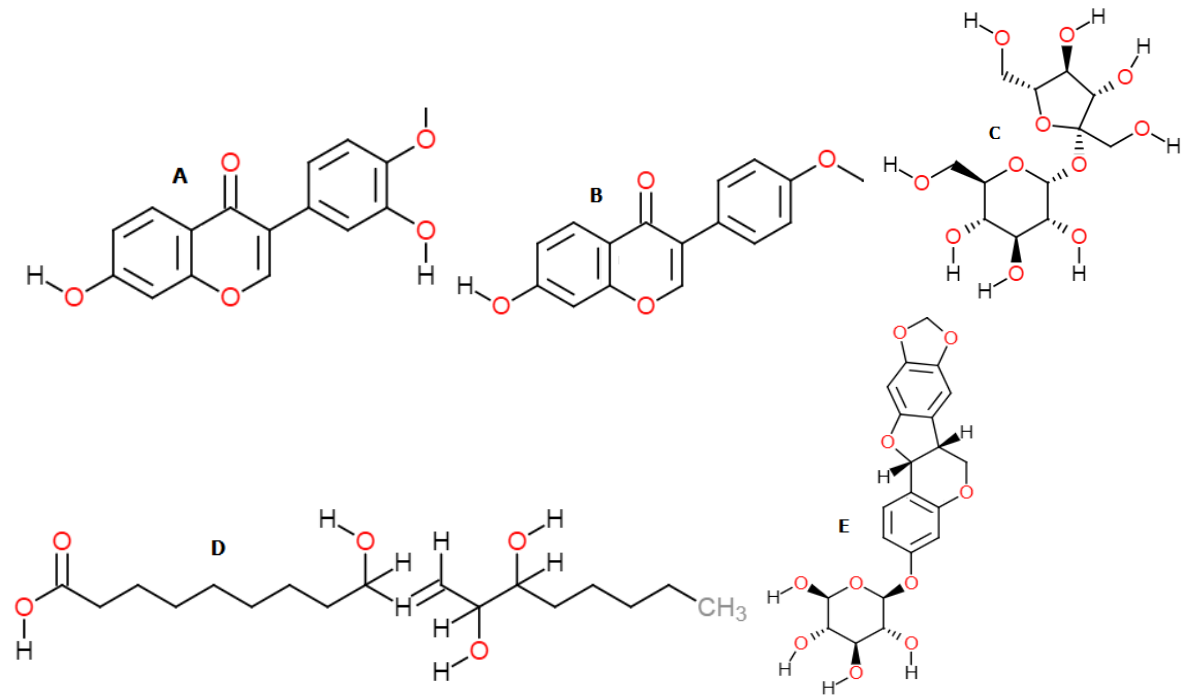


Fig. 39 Structures of some of the main compounds identified from crude herbal extracts of *A.membranaceus*: (A) Calycosin; (B) Formononetin; (C) Example of a dihexose sugar structure; (D) Trihydroxy octadecenoic acid; (E) Trifolirhizin.

Table [13] Compounds detected in *O.basilicum*, *I.helenium*, *W.somnifera* and *A.membranaceus* extracts in negative scan mode in LC-MS/ PDA.

SL NO.	RT (MIN)	M-H	$[M-H]^-$	MS/MS[C]	TENTATIVE ID[#]	COMPOUND NATURE
ObA1						
1	21.65	359.0736	$C_{18}H_{15}O_8$	161, 133, 135, 179, 197	**Rosmarinic acid**	Phenolic acid
2	1.75	149.0076	$C_4H_5O_6$	113, 130.997, 141	**Tartaric acid**	Organic acid
3	3.86	191.0175	$C_6H_7O_7$	111, 129, 173	**Isocitric acid**	Citric acid
4	24.36	327.2150	$C_{18}H_{15}O_6$	116.9, 205, 215, 277, 311	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavones
5	10.59	311.0392	$C_{13}H_{11}O_9$	135, 149, 179, 311	**Caftaric acid (Caffeoyl-tartaric acid)**	Non-flavanoid phenolic
8	16.22	225.1110	$C_{12}H_{17}O_4$	112, 135, 161, 195, 203, 216	**12-hydroxyjasmonic acid**	Carboxylic acid
6	18.23	473.0708	$C_{22}H_{17}O_{12}$	135, 149, 179, 293, 311	**Chicoric acid (dicaffeoyl-tartaric acid)**	Hydroxycinnamic acid
7	23.38	717.1448	$C_{36}H_{29}O_{16}$	243, 343, 519	***Unknown***	–
ObM2						
1	21.61	359.0761	$C_{18}H_{15}O_8$	161, 133, 135, 179, 197	**Rosmarinic acid**	Phenolic acid
2	1.79	341.1076	$C_{12}H_{21}O_{11}$	89, 173	**Dihexose**	Sugar
3	24.36	327.2150	$C_{18}H_{15}O_6$	116.9, 205, 215, 277, 311	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavones
4	18.09	609.1499	$C_{27}H_{29}O_{16}$	151, 255, 271, 300, 301	**Rutin (Quercetin-hexoside-rhamnoside)**	Flavonoid
5	14.45	387.1648	$C_{21}H_{23}O7$	59, 119, 207, 300	**Medioresinol**	Furanoid lignin
6	18.74	463.0882	$C_{21}H_{19}O_{12}$	89, 151, 255, 271, 300	**Isoquercetin (Quercetin-hexoside)**	Flavonoid
7	23.38	717.1450	$C_{36}H_{29}O_{16}$	243, 343, 519	***Unknown***	–
8	20.99	137.1212	$C_9H_{13}O$	93, 121	**trans-Ocimene oxide**	Monoterpenes
ObE3						
1	21.6	359.0758	$C_{18}H_{15}O_8$	161, 133, 135, 179, 197	**Rosmarinic acid**	Phenolic acid
2	9.35	153.4201	$C_{10}H_{17}O$	79, 93, 121, 136	**Linalool (2,6-Dimethyl-2,7-octadien-6-ol; allo-Ocimenol)**	Terpene alcohol
3	24.36	327.2150	$C_{18}H_{15}O_6$	116.9, 205, 215, 277, 311	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavone
4	14.76	431.1907	$C_{21}H_{19}O_{10}$	153, 205, 269, 354, 385	**Apigenin-7-O-glucoside**	Flavonoid glycoside
5	13.82	179.0330	$C_9H_7O_4$	135	**Caffeic acid**	Hydroxycinnamic acid
6	24.43	329.2310	$C_{18}H_{33}O_5$	171, 211	**Trihydroxy octadecenoic acid**	Fatty acid
7	21.15	187.0958	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cyclopentene dimethanol
8	16.19	225.1113	$C_{12}H_{17}O_4$	112, 135, 161, 195, 203, 216	**12-hydroxyjasmonic acid**	Carboxylic acid
9	18.71	463.0874	$C_{21}H_{19}O_{12}$	89, 151, 255, 271, 300	**Isoquercetin (Quercetin-hexoside)**	Flavonoid
IhA1						
1	1.79	341.1082	$C_{12}H_{21}O_{11}$	89, 179, 267	**Dihexose**	Sugar
2	1.71	317.0541	$C_{18}H_{23}O_5$	80, 96, 164, 225	**10-isobutyryloxy-8,9-epoxythymol isobutyrate**	Isobutyrate
3	3.88	191.0173	$C_6H_7O_7$	111, 129, 173	**Isocitric acid**	Citric acid
4	26.30	555.2842	$C_{27}H_{37}O_{10}$	112.98, 180.97, 248, 316, 384.9	**(E)-Fukugetin**	Flavonoid
5	1.33	173.1028	$C_9H_{17}O3$	131	***Unknown***	–
6	10.50	353.0718	$C_{16}H_{17}O_9$	179, 191	**Chlorogenic Acid (3-Caffeoylquinic acid)**	Polyphenols/flavonoids

7	13.12	353.0862	$C_{16}H_{17}O_9$	173, 179, 191	**Cryptochlorogenic Acid (4-Caffeoylquinic acid)**	Polyphenols/flavonoids
8	12.72	353.0858	$C_{16}H_{17}O_9$	191	**5-Caffeoylquinic acid**	Polyphenols/flavonoids
9	5.90	827.2750	$C_{41}H_{47}O_{18}$	89, 179, 341, 503, 665	***Unknown***	–
10	19.59	457.2078	$C_{30}H_{40}O_4$	112, 191, 209, 371	**Epigallocatechin gallate**	Polyphenols/flavonoids
11	24.53	233.1176	$C_{14}H_{17}O_3$, $C_{10}H_{17}O_6$	116, 161, 213	**Unknown**	–
12	24.87	247.1327	$C_{15}H_{19}O_3$	115.92, 197, 221, 233	**5alpha-Epoxyalantolactone**	Lactone
IhM2						
1	24.77	293.1378	$C_{17}H_{25}O_4$	87, 116, 231	**Tanacetol A (2-Acetoxy-11-hydroxy-1(10),4(15)-germacradien-5-one)**	Germacrane sesquiterpenoids
2	24.44	329.2317	$C_{18}H_{33}O_5$	116, 171, 239	**Trihydroxy octadecenoic acid**	Fatty acid
3	22.95	239.1279	$C_{13}H_{19}O_4$	161, 177	***Unknown***	–
4	19.56	457.2078	$C_{22}H_{17}O_{11}$	112, 191, 209, 371	**Epigallocatechin Gallate**	Polyphenols/flavonoids
5	24.22	693.4105	$C_{45}H_{57}O_6$	114, 201, 346	***Unknown***	–
6	26.30	265.1459	$C_{15}H_{22}O_4$	112, 180	**Macrophyllilactone B**	Lactone
7	21.09	187.0958	$C_9H_{15}O_4$	113, 123, 143, 170	***Unknown***	–
8	12.71	191.0552	$C_7H_{11}O_6$	85, 135, 161, 179	**Chlorogenic acid derivative**	Polyphenols
IhE3						
1	24.77	293.1382	$C_{17}H_{25}O_4$	87, 116, 231	**Tanacetol A (2-Acetoxy-11-hydroxy-1(10),4(15)-germacradien-5-one)**	Germacrane sesquiterpenoids
2	26.30	265.1458	$C_{15}H_{22}O_4$	112.98, 183, 248.95	**Macrophyllilactone B**	Lactone
3	22.95	239.1271	$C_{13}H_{19}O_4$	97, 161, 177	***Unknown***	–
4	24.45	329.2312	$C_{18}H_{33}O_5$	59, 116, 171, 187, 239	**Trihydroxy octadecenoic acid**	Fatty acid
5	21.11	187.0959	$C_9H_{15}O_4$	113, 123, 143, 170	***Unknown***	–
6	19.57	457.2078	$C_{22}H_{17}O_{11}$	112, 191, 209, 371	**Epigallocatechin Gallate**	Polyphenols/flavonoids
7	21.43	225.1149	$C_{11}H_{13}O_5$	125, 163, 169	**Trimethoxybenzoic acid methyl ester**	Acid ester
8	12.71	191.0535	$C_7H_{11}O_6$	85, 135, 161, 179	**Chlorogenic acid derivative**	Polyphenols
IhEtA4						
1	24.77	293.1382	$C_{17}H_{25}O_4$	87,116, 231	**Tanacetol A (2-Acetoxy-11-hydroxy-1(10),4(15)-germacradien-5-one)**	Germacrane sesquiterpenoids
2	26.30	265.1459	$C_{15}H_{22}O_4$	112.98, 183, 248.95	**Macrophyllilactone B**	Lactone
3	22.96	239.1271	$C_{13}H_{19}O_4$	97,161, 177	***Unknown***	–
4	24.43	329.2318	$C_{18}H_{33}O_5$	59, 116,171,187, 239	**Trihydroxy octadecenoic acid**	Fatty acid
5	21.13	187.0959	$C_9H_{15}O_4$	113, 123, 143, 170	***Unknown***	–
6	19.58	457.2078	$C_{22}H_{17}O_{11}$	112, 191, 209, 371	**Epigallocatechin Gallate**	Polyphenols/flavonoids
7	21.45	225.1149	$C_{11}H_{13}O_5$	125,163,169	**Trimethoxy benzoic acid methyl ester**	Acid ester
8	20.06	239.1273	$C_{13}H_{19}O_4$	125,161,177,193	***Unknown***	–
WsM2						
1	24.36	811.4113	$C_{51}H_{89}O_7$	179, 329, 503, 765	***Unknown***	–
2	24.12	827.4061	$C_{41}H_{63}O_{17}$, $C_{48}H_{59}O_{12}$	179, 312, 503, 653, 781	***Unknown***	–
3	24.57	327.2144	$C_{18}H_{15}O_6$	116.9, 205, 215, 277, 311	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavone
4	1.81	341.1064	$C_{12}H_{21}O_{11}$	89, 179, 267	**Dihexose**	Sugar
5	21.68	1093.5665	$C_{56}H_{81}O_{25}$	341, 763, 932	**Pentagycoside complex[?]**	Glycoside

6	2.11	133.0129	C_8H_7NO	43, 71, 93	**Isopelletierine (1-Pyridin-2-ylpropan-2-one)**	Alkaloid
7	13.09	472.0012	$C_{28}H_{39}O_6$	186, 308, 350	**Withaferin A, dihydro- (2,3-Dihydrowithaferin-A)**	Steroidal Lactone
WsEtA4						
1	24.28	533.2756	$C_{29}H_{41}O_9$	89, 179, 327	**Withaperuvin I**	Withanolide
2	24.36	811.4103	$C_{51}H_{89}O_7$	179, 329, 503, 765	***Unknown***	–
3	24.43	329.2314	$C_{18}H_{33}O_5$	116.92, 171	**Trihydroxy octadecenoic acid**	Fatty acid
4	23.19	567.2249	$C_{34}H_{50}NO_6$	96, 179, 341, 503	**Withaferin derivative (Withaferin deriv GG-AM-4)**	Steroidal Lactone
5	21.12	187.0954	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cyclopentene dimethanol
AmM2						
1	1.79	341.1072	$C_{12}H_{21}O_{11}$	89, 101, 119, 161, 179	**Dihexose**	Sugar
2	24.43	329.2312	$C_{18}H_{33}O_5$	116.92, 171, 211, 258	**Trihydroxy octadecenoic acid**	Fatty acid
3	24.23	283.0591	$C_{16}H_{11}O_5$	136, 196, 211, 240, 269	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
4	24.57	267.0653	$C_{16}H_{11}O_4$	115,117,196, 207, 236	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
5	23.23	567.2245	$C_{28}H_{39}O_{10}S$	96,179, 341, 503	***Unknown***	–
6	18.03	491.1185	$C_{22}H_{22}O_{10}$	233, 255, 268	**Trifolirhizin ((-)-Maackiain-3-Oglucoside)**	Flavonoid
7	6.52	383.1220	$C_{14}H_{23}O_{12}$	156, 323	***Unknown***	–
8	21.16	187.0963	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cyclopentene dimethanol
AmE3						
1	24.22	283.0591	$C_{16}H_{11}O_5$	136, 196, 211, 240, 269	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
2	24.57	267.0653	$C_{16}H_{11}O_4$	115, 117, 196, 207, 236	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
3	1.80	341.1072	$C_{12}H_{21}O_{11}$	89, 101, 119, 161, 179	**Dihexose**	Sugar
4	24.43	329.2322	$C_{18}H_{33}O_5$	116.92, 171, 211, 258	**Trihydroxy octadecenoic acid**	Fatty acid
5	18.02	491.1198	$C_{22}H_{22}O_{10}$	233, 255, 268	**Trifolirhizin ((-)-Maackiain-3-Oglucoside)**	Flavonoid
6	23.01	475.1220	$C_{23}H_{23}O_{11}$	267, 343	***Unknown***	–
7	21.12	187.0936	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cyclopentene dimethanol
AmEtA4						
1	24.22	283.0591	$C_{16}H_{11}O_5$	136, 196, 211, 240, 269	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
2	24.57	267.0367	$C_{16}H_{11}O_4$	115, 117, 196, 207, 236	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
3	24.43	329.2317	$C_{18}H_{33}O_5$	116.92, 171, 211, 258	**Trihydroxy octadecenoic acid**	Fatty acid
4	24.83	311.2206	$C_{18}H_{31}O_4$	117, 183, 223	***Unknown***	–
5	24.89	913.4787	$C_{46}H_{73}O_{18}$	311, 402, 708	***Unknown***	–

[#]**REFERENCES** – Stander et al., 2017; Said et al., 2017; Chen et al., 2017; Chang et al., 2016; Chen et al., 2016; Zhang et al., 2016; Liu et al., 2016; Simirgiotis et al., 2015; Du et al., 2014; Kuo et al., 2014; Zhang et al., 2013; Mena et al., 2012; Wang et al., 2012; Čejchanová, 2011; Hossain et al., 2010.
https://www.ncbi.nlm.nih.gov/pccompound; https://massbank.eu/MassBank/; https://metlin.scripps.edu/; https://webbook.nist.gov/chemistry/mw-ser/

For the positive mode extract scans, the following observations were noted (details summarized in table 14):

1. *Ocimum basilicum* (aqueous, methanol and ethanol) extracts (fig. 40 shows the chromatogram of the ethanol extract):

- The most prominent peak in the methanol and ethanolic extracts of *O.basilicum* was salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone) with a retention time of 7.32 min at *m/z* 329 at a PDA detection wavelength 230 nm.
- Pyridine carboxylic acid esters (fig. 41 b) were identified in all extracts at *m/z* 398 and product ions at *m/z* 149, 240 and 266.
- Other compounds include the flavone eupatorin (3',5-dihydroxy-4',6,7-trimethoxyflavone, 345 *m/z*) (fig. 41 a) and the aromatic lactone furan-2(3H)-one complex (311 *m/z*) at retention times 6.42 and 6.91 min, respectively.

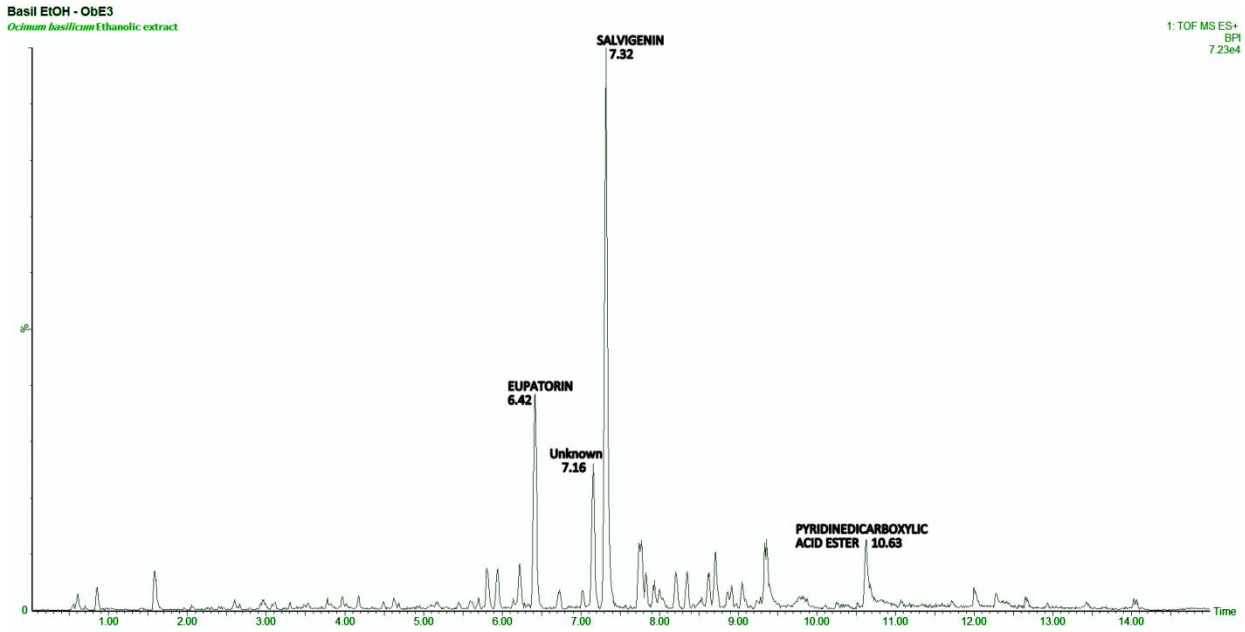


Fig. 40 LC-MS chromatogram of *O.basilicum* (ethanol extract) in positive mode scan for 15 min.

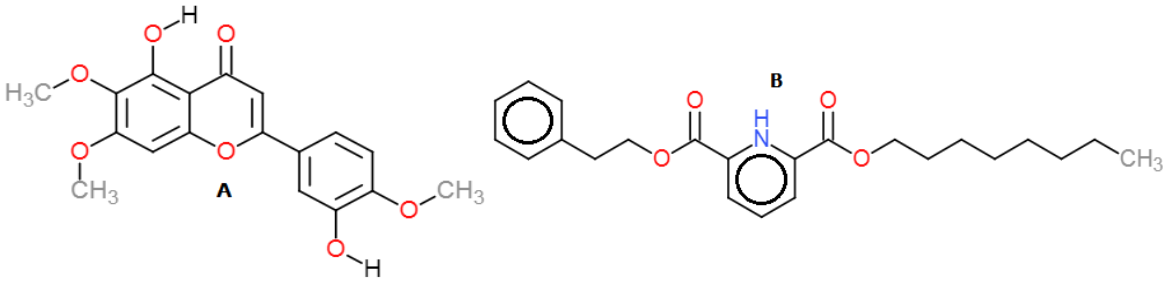


Fig. 41 Structures of some of the main compounds identified from crude herbal extracts of *O.basilicum* (positive mode): (A) Eupatorin; (B) Pyridine carboxylic acid ester.

2. *Inula helenium* (aqueous, methanol, ethanol and ethyl acetate) extracts:
   - The most prominent peak observed in all the organic solvent extracts of *I.helenium* was the known sesquiterpene lactone helenin (isoalantolactone) with a retention time of 8.33 min at *m/z* 465 at a PDA detection wavelength 230 nm (fig. 42 a). The product ions were noted at *m/z* 105, 151, 187, 215 and 233.

- Other compounds included pyridine carboxylic acid ester at *m/z* 398 and apigenin at *m/z* 271.
- Major peaks in the methanolic extract detected at 4.78 and 4.9 min (*m/z* 348 and 304), and the ethyl acetate extract at 6.12 and 8.21 min (*m/z* 271 and 465) could not be identified.

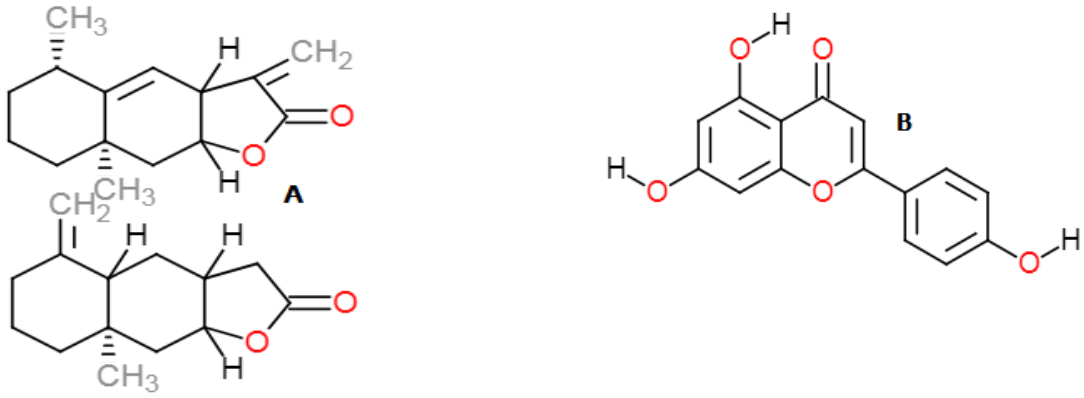


Fig. 42 Structures of some of the main compounds identified from crude herbal extracts of *I.helenium* (positive mode): (A) Helenin (isoalantolactone); (B) Apigenin.

3. *Withania somnifera* (methanol and ethyl acetate) extracts:
   - The major withanolides were observed in all extracts of *W.somnifera* in the positive mode scan. Withanolide A and withanolide B (fig. 43 a, b) were identified with retention times 5.54 and 7.53 min at *m/z* 471 and 455 respectively, at a PDA detection wavelength 230 nm. The product ions were noted as *m/z* 125, 246, 276, 299 and *m/z* 401, 419, 437 respectively.
   - Withaferin A was identified at retention time 5.92 min and *m/z* 471 (fig. 43 c) and product ions at *m/z* 263, 265, 283, 353, 417 and 435.
   - Other known compounds observed were vicosalactone B (489 *m/z*), withanamide D/E (783 *m/z*) and withasomidienone (439 *m/z*).

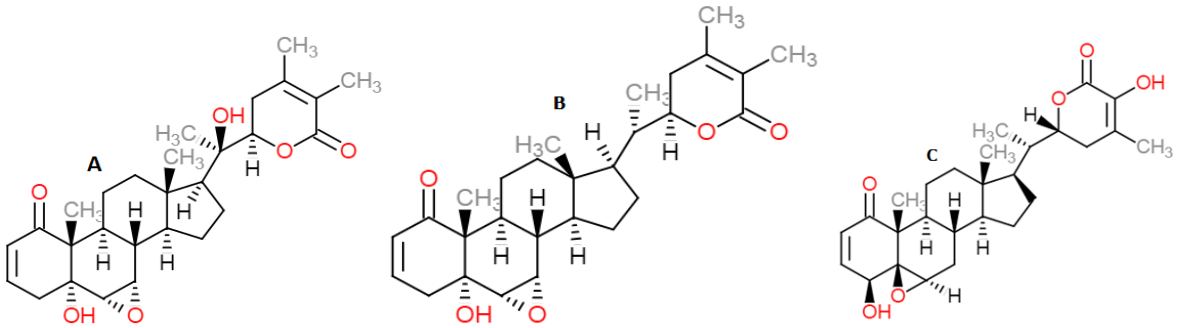


Fig. 43 Structures of some of the main compounds identified from crude herbal extracts of *W.somnifera* (positive mode): (A) Withanolide A; (B) Withanolide B; (C) Withaferin A.

4. *Astragalus membranaceus* (methanol, ethanol and ethyl acetate) extracts:

- The major known compounds within *A.membranaceus* were observed in the positive mode scan. Astragaloside IV and astragaloside I (fig. 44 a, b) were identified with retention times 5.61 and 6.87 min at *m/z* 785 and 869 respectively, at a PDA detection wavelength 230 nm. The product ions were noted as *m/z* 269, 437, 587 and *m/z* 143, 297, 437, 455, 653, 671, respectively.
- Other major compounds observed included formononetin (269 *m/z*), calycosin (285 *m/z*), acetylastragaloside I (911 *m/z*), astragaloside II (827 *m/z*) and ononin (431 *m/z*) at 230nm.
- The benzopyran derivative 3',4',7-trimethoxyflavan and the carboxylic acid 4,10-dihydroxy-3,9-dimethoxypterocarpan, was observed in the ethyl acetate extract at retention times 9.35 min and 7.03 min respectively.

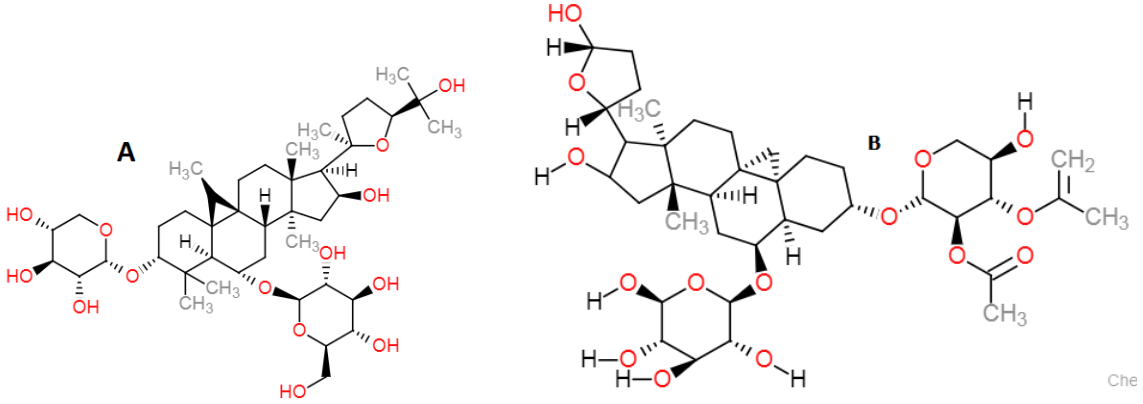


Fig. 44 Structures of some of the main compounds identified from crude herbal extracts of *A.membranaceus* (positive mode): (A) Astragaloside IV; (B) Astragaloside I.

Table [14] Secondary metabolites/compounds detected in *O.basilicum*, *I.helenium*, *W.somnifera* and *A.membranaceus* extracts in positive scan mode in LC-MS/ PDA.

SL NO.	RT (MIN)	M+H	$[M+H]^+$	MS/MS[C]	TENTATIVE ID[#]	TYPE OF COMPOUND
ObA1						
1	10.63	398.2331	$C_{24}H_{32}NO_4$	149, 240, 266	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
2	7.74	304.3008	$C_{21}H_{38}N$	91, 212	**Pyridine/ Amine complex**	Secondary Metabolites
3	12.28	411.2662	$C_{30}H_{35}O$	175, 283, 355	***Unknown***	–
4	6.91	311.2210	$C_{18}H_{31}O_4$	96, 149, 219, 275	**Furan-2(3H)-one complex**	Aromatic lactone
5	9.06	399.2498	$C_{25}H_{35}O_4$, $C_{18}H_{39}O_9$	221, 324	***Unknown***	–
ObM2						
1	7.32	329.1014	$C_{18}H_{17}O_6$	133, 268	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavones
2	10.63	398.2324	$C_{24}H_{32}NO_4$	149, 240, 266	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
3	7.74	304.2997	$C_{21}H_{38}N$	91, 212	**Pyridine/ Amine complex**	Secondary Metabolites
4	6.42	345.0979	$C_{18}H_{17}O_7$	182, 240, 312, 315	**Eupatorin (3',5-Dihydroxy-4',6,7-trimethoxyflavone)**	Flavones
ObE3						
1	7.32	329.1023	$C_{18}H_{17}O_6$	133, 268	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavones

2	6.42	345.0959	$C_{18}H_{17}O_7$	182, 240, 312, 315	**Eupatorin (3',5-Dihydroxy-4',6,7-trimethoxyflavone)**	Flavones
3	7.16	445.2117	$C_{21}H_{33}O_{10}$, $C_{32}H_{29}O_2$	105, 194 224, 385, 407	***Unknown***	–
4	10.63	398.2336	$C_{24}H_{32}NO_4$	149, 240, 266	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
IhA1						
1	10.62	398.3220	$C_{24}H_{32}NO_4$	149, 240, 266	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
2	12.28	411.2646	$C_{30}H_{35}O$, $C_{19}H_{39}O_9$	175, 231, 283, 355	***Unknown***	–
IhM2						
1	4.78	348.2174	$C_{20}H_{28}O_5$	128, 302, 304	***Unknown***	–
2	4.90	304.2271	$C_{18}H_{13}N_3O_2$	84, 128	***Unknown***	–
3	8.34	465.3008	$C_{30}H_{41}O_4$	105, 233, 383	**Helenin : Isoalantolactone**	Sesquiterpene lactones
IhE3						
1	8.33	465.3002	$C_{30}H_{41}O_4$	105, 151, 187, 215, 233	**Helenin : Isoalantolactone**	Sesquiterpene lactones
2	6.12	271.1299	$C_{15}H_{11}O_5$	105, 121, 131, 182, 231, 240	**Apigenin**	Flavone
3	8.20	465.2986	$C_{30}H_{41}O_4$	314, 443	***Unknown***	–
4	10.62	398.2334	$C_{24}H_{32}NO_4$	149, 240, 266	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
IhEtA4						
1	8.34	465.3006	$C_{30}H_{41}O_4$	105, 151, 187, 215, 233	**Helenin : Isoalantolactone**	Sesquiterpene lactones
2	6.12	271.1334	$C_{17}H_{19}O_3$	185, 231	***Unknown***	–
3	8.21	465.2995	$C_{30}H_{41}O_4$	314, 443	***Unknown***	–
4	9.36	301.1416	$C_{18}H_{21}O_4$, $C_{11}H_{25}O_9$	149, 231	***Unknown***	–
WsM2						
1	5.54	471.2741	$C_{28}H_{39}O_6$	125, 246, 276, 299	**Withanolide A**	Secondary metabolite
2	5.92	471.2727	$C_{28}H_{39}O_6$	263, 265, 283, 353, 417, 435	**Withaferin A**	Steroidal Lactone
3	5.69	503.3006	$C_{29}H_{43}O_7$	331, 407, 425, 443	**2,25-Diepoxywithaferin A**	Steroidal Lactone
4	7.54	455.2797	$C_{28}H_{39}O_5$	401, 419, 437	**Withanolide B**	Secondary metabolite
5	4.71	489.2848	$C_{28}H_{41}O_7$	281, 299, 317	**Vicosalactone B**	Lactone
6	4.38	783.4157	$C_{40}H_{67}N_2O_{13}$	441, 459, 621	**Withanamide D/E**	Secondary metabolite
7	3.79	754.3456	$C_{38}H_{62}N_2O_{13}$	574, 593, 630, 736	**Withanamide B/C isomer**	Secondary metabolite
WsEtA4						
1	5.54	471.2744	$C_{28}H_{39}O_6$	125, 246, 276, 299	**Withanolide A**	Secondary metabolite
2	5.92	471.2742	$C_{28}H_{39}O_6$	263, 265, 283, 353, 417, 435	**Withaferin A**	Steroidal Lactone
3	7.53	455.2791	$C_{28}H_{39}O_5$	401, 419, 437	**Withanolide B**	Secondary metabolite
4	5.70	503.3001	$C_{29}H_{43}O_7$	331, 407, 425, 443	**2,25-Diepoxywithaferin A**	Steroidal Lactone
5	4.71	489.285	$C_{28}H_{41}O_7$	317, 459	**Vicosalactone B**	Lactone
6	7.22	439.2825	$C_{28}H_{39}O_4$	187, 367, 383, 427	**Withasomidienone**	Secondary metabolite
AmM2						
1	5.61	785.4658	$C_{41}H_{68}O_{14}$	269, 437, 587	**Astragaloside IV**	Triterpene glycoside
2	5.65	269.0811	$C_{16}H_{13}O_4$	71, 190, 197, 253	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
3	4.44	285.0765	$C_{16}H_{13}O_5$	137, 241, 270	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
4	10.63	398.2325	$C_{24}H_{32}NO_4$	149, 240, 266, 296	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
5	3.05	447.1285	$C_{22}H_{23}O_{10}$	285, 365, 429	**Calycosin 7-O-beta-D-glucoside**	Isoflavone glucoside
6	6.87	869.9849	$C_{45}H_{73}O_{16}$	143, 297, 437, 455, 653, 671	**Astragaloside I**	Triterpene glycoside

7	3.91	489.1394	$C_{24}H_{25}O_{11}$, $C_{17}H_{29}O_{16}$	149, 270, 285	***Unknown***	–
8	4.09	431.1328	$C_{22}H_{23}O_9$	268.2	**Ononin**	Isoflavone glycoside
AmE3						
1	5.65	269.0803	$C_{16}H_{13}O_4$	71, 190, 197, 253	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
2	5.60	785.4661	$C_{41}H_{68}O_{14}$	269, 437, 587	**Astragaloside IV**	Triterpene glycoside
3	4.43	285.0757	$C_{16}H_{13}O_5$	137, 241, 270	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
4	6.88	869.9915	$C_{45}H_{73}O_{16}$	143, 297, 437, 455, 653, 671	**Astragaloside I**	Triterpene glycoside
5	9.34	301.1404	$C_{18}H_{21}O_4$, $C_{11}H_{25}O_9$	121, 149, 150	***Unknown***	–
6	8.17	911.4993	$C_{47}H_{75}O_{17}$	437, 695, 753	**Acetylastragaloside I**	Triterpene glycoside
7	10.62	398.2334	$C_{24}H_{32}NO_4$	149, 240, 266, 296	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
8	6.04	827.9796	$C_{43}H_{71}O_{15}$	629, 707 809	**Astragaloside II**	Triterpene glycoside
9	3.91	489.1394	$C_{24}H_{25}O_{11}$, $C_{17}H_{29}O_{16}$	149, 270, 285	***Unknown***	–
10	4.09	431.1331	$C_{22}H_{23}O_9$	268.2	**Ononin**	Isoflavone glycoside
AmEtA4						
1	5.65	269.0810	$C_{16}H_{13}O_4$	71, 190, 197, 253	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
2	4.43	285.0764	$C_{16}H_{13}O_5$	137, 241, 270	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
3	6.68	869.9904	$C_{45}H_{73}O_{16}$	143, 297, 437, 455, 653, 671	**Astragaloside I**	Triterpene glycoside
4	9.35	301.1416	$C_{17}H_{17}O_5$	121, 149, 150	**9,10-Dimethoxy-6a,11a-dihydro-6H-[1]benzofuro[3,2-c]chromen-3-ol**	Benzopyran derivative
5	10.62	398.2323	$C_{24}H_{32}NO_4$	149, 240, 266, 296	**2,6-Pyridinedicarboxylic acid, nonyl phenethyl ester**	Carboxylic acid
6	7.03	316.9838	$C_{17}H_{17}O_6$	149, 280, 298	**4,10-Dihydroxy-3,9-dimethoxypterocarpan**	Flavonoid
7	3.91	489.1419	$C_{24}H_{25}O_{11}$, $C_{17}H_{29}O_{16}$	149, 270, 285	***Unknown***	–
8	4.09	431.1339	$C_{22}H_{23}O_9$	268.2	**Ononin**	Isoflavone glycoside

[#]**REFERENCES** – Liu et al., 2018; Chang et al., 2016; Liu et al., 2016; Du et al., 2014; Wang et al., 2012; Bolleddula et al., 2012; Čejchanová, 2011; Hossain et al., 2010; Ye et al., 2005.
https://www.ncbi.nlm.nih.gov/pccompound; https://massbank.eu/MassBank/; https://metlin.scripps.edu/; https://webbook.nist.gov/chemistry/mw-ser/

The major identified compounds for the twelve active extracts showing inhibition, were quantified (in mg/L equivalent units of gallic acid/ quercetin) relative to the linear calibration curve of the reference standards - gallic acid for all the acid compounds and quercetin for the non-acid compounds (table 15).

Table [15] Relative quantification of the identified phytocompounds detected in *O.basilicum*, *I.helenium*, *W.somnifera* and *A.membranaceus* extracts in positive scan mode in LC-MS/ PDA.

# SLNO	COMPOUND NAME	SAMPLE NAME	AREA	RT(MIN)	CONC. UNITS * (MG/L EQUIVALENTS OF GALLIC ACID/ QUERCETIN)
Compound 1	**Rosmarinic acid**				**
1		**ObA1**	1699.1760	21.61	2298.037
2		**ObM2**	1423.3300	21.61	1923.756
3		**ObE3**	585.0240	21.61	786.304
Compound 2	**Tartaric acid**				
1		**ObA1**	593.9001	1.76	798.347
2		**ObM2**	33.9320	1.76	38.556

3		**ObE3**	45.3010	1.76	53.982
Compound 3	**Isocitric acid**				
1		**ObA1**	742.4790	3.88	999.946
2		**IhA1**	742.2550	3.88	999.642
3		**WsM2**	34.6080	3.89	39.474
4		**AmM2**	94.1240	3.88	120.228
Compound 4	**Caftaric acid**				
1		**ObA1**	407.1970	10.60	545.019
Compound 5	**Chicoric acid**				
1		**ObA1**	371.2370	18.23	496.228
2		**ObM2**	22.3180	18.26	22.798
Compound 6	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**				*******
1		**ObA1**	430.9610	24.36	73.634
2		**ObM2**	213.4770	24.36	24.150
3		**ObE3**	670.6350	24.36	128.167
4		**IhM2**	90.6170	24.37	0.020
5		**IhE3**	85.7520	24.36	0.015
6		**IhEtA4**	103.6490	24.36	0.035
7		**AmM2**	169.7220	24.36	14.194
8		**AmE3**	225.7240	24.36	26.936
9		**AmEtA4**	266.1060	24.36	36.124
Compound 7	**Rutin (Quercetin-Hexoside-rhamnoside)**				
1		**ObA1**	80.0950	18.07	0.010
2		**ObM2**	144.1280	18.09	8.371
3		**ObE3**	146.4550	18.07	8.900
Compound 8	**Isoquercetin (Quercetin-Hexoside)**				
1		**ObM2**	108.5580	18.73	0.277
2		**ObE3**	156.3420	18.72	11.150
Compound 9	**Linalool (2,6-Dimethyl-2,7-octadien-6-ol)**				
1		**ObE3**	133.6801	9.35	5.993
Compound 10	**12-hydroxyjasmonic acid**				
1		**ObA1**	262.7230	16.21	348.991
2		**ObE3**	255.5850	16.19	339.305
Compound 11	**Caffeic acid**				
1		**ObM2**	29.9890	13.83	33.206
2		**ObE3**	433.6770	13.80	580.949
3		**IhM2**	69.7520	13.79	87.159
4		**IhE3**	29.0440	13.78	31.924
5		**IhEtA4**	47.1690	13.78	56.517
Compound 12	**5-Caffeoylquinic acid**				
1		**IhA1**	249.3501	12.72	330.846
2		**IhM2**	138.6160	12.70	180.596
3		**IhE3**	148.7140	12.69	194.298
4		**IhEtA4**	61.1370	12.69	75.469
5		**WsM2**	30.0660	12.73	33.311

Compound 13	**3-Caffeoylquinic acid**				
1		**IhA1**	27.0460	10.50	29.213
2		**IhM2**	5.6830	10.47	0.227
Compound 14	**4-Caffeoylquinic acid**				
1		**IhA1**	43.5890	13.12	51.659
Compound 15	**(E)-Fukugetin**				
1		**ObE3**	45.8240	26.32	0.005
2		**IhA1**	80.2310	26.31	0.010
3		**IhM2**	41.0590	26.32	0.005
Compound 16	**Medioresinol**				
1		**ObM2**	114.7220	14.45	6.020
2		**ObE3**	69.9301	14.44	0.005
Compound 17	**Apigenin-7-O-glucoside**				
1		**ObM2**	75.4170	14.77	0.010
2		**ObE3**	404.7730	14.75	67.675
3		**WsM2**	54.0010	14.77	0.005
4		**WsEtA4**	43.6480	14.75	0.004
Compound 18	**Epigallocatechin Gallate**				
1		**IhA1**	124.1370	19.58	3.822
2		**IhM2**	862.2060	19.56	171.755
3		**IhE3**	282.0750	19.56	39.758
4		**IhEtA4**	203.1610	19.58	21.802
Compound 19	**Chlorogenic acid derivative(quinic acid isomer)**				
1		**IhA1**	258.4760	12.72	343.228
2		**IhM2**	151.3150	12.71	197.827
3		**IhE3**	158.6690	12.7	207.805
4		**IhEtA4**	71.3690	12.7	89.353
Compound 20	**5alpha-Epoxyalantolactone**				
1		**IhA1**	38.9350	24.87	0.003
2		**IhE3**	36.9050	24.86	0.003
Compound 21	**Tanacetol A (2-Acetoxy-11-hydroxy-1(10),4(15)-germacradien-5-one)**				
1		**IhA1**	261.0970	24.79	34.985
2		**IhM2**	1732.9920	24.77	369.886
3		**IhE3**	1067.0550	24.77	218.365
4		**IhEtA4**	958.1890	24.77	193.594
Compound 22	**Macrophyllilactone B**				
1		**IhM2**	850.4980	26.27	169.091
2		**IhE3**	1328.5220	26.27	277.856
3		**IhEtA4**	1476.2560	26.28	311.470
Compound 23	**Trihydroxy octadecenoic acid**				
1		**ObA1**	127.4110	24.44	165.393
2		**ObM2**	100.3910	24.44	128.731
3		**ObE3**	396.4770	24.44	530.474
4		**IhA1**	121.5620	24.44	157.457
5		**IhM2**	776.1910	24.44	1045.688

6		**IhE3**	357.1530	24.44	477.118
7		**IhEtA4**	369.2920	24.44	493.588
8		**WsM2**	737.4840	24.44	993.168
9		**WsEtA4**	887.2440	24.44	1196.370
10		**AmM2**	565.2730	24.44	759.505
11		**AmE3**	929.1930	24.44	1253.288
12		**AmEtA4**	857.0140	24.43	1155.352
Compound 24	**Isopelletierine (1-Pyridin-2-ylpropan-2-one)**				
1		**IhA1**	344.2740	2.14	53.910
2		**WsM2**	936.7730	2.14	188.721
3		**AmM2**	187.9380	2.16	18.339
4		**AmE3**	55.1380	2.15	0.005
Compound 25	**2,3-Dihydrowithaferin-A**				
1		**WsM2**	172.1430	13.09	14.745
Compound 26	**Withaperuvin I**				
1		**WsM2**	719.6570	24.29	139.321
2		**WsEtA4**	1141.7950	24.29	235.370
Compound 27	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**				
1	**- ^{ve} scan mode**	**AmM2**	474.4250	24.22	83.523
2		**AmE3**	1356.2370	24.22	284.162
3		**AmEtA4**	1106.9550	24.22	227.443
1	**+^{ve} scan mode**	**AmM2**	450.4570	4.44	230.881
2		**AmE3**	1335.9570	4.44	684.621
3		**AmEtA4**	969.9740	4.44	497.087
Compound 28	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**				
1	**- ^{ve} scan mode**	**AmM2**	358.6960	24.57	57.191
2		**AmE3**	962.8320	24.57	194.651
3		**AmEtA4**	889.0850	24.57	177.871
4	**+^{ve} scan mode**	**AmM2**	429.2270	5.65	220.003
5		**AmE3**	1402.4480	5.65	718.691
6		**AmEtA4**	1218.4690	5.65	624.419
Compound 29	**Eucommiol**				
1		**ObA1**	138.4490	21.16	7.078
2		**ObE3**	354.5340	21.15	56.244
3		**IhM2**	402.1650	21.12	67.082
4		**IhE3**	299.7260	21.12	43.774
5		**IhEtA4**	399.0440	21.13	66.372
6		**WsM2**	210.6120	21.14	23.498
7		**WsEtA4**	321.8020	21.12	48.797
8		**AmM2**	98.4510	21.15	0.019
9		**AmE3**	140.9520	21.12	7.648
Compound 30	**Dihexose**				
1		**ObM2**	279.5990	1.8	39.194
2		**IhA1**	695.6030	1.8	133.848
3		**IhM2**	164.2950	1.79	12.959
4		**IhE3**	110.4890	1.79	0.717

5		**WsM2**	1317.3660	1.81	275.318
6		**WsEtA4**	209.1890	1.8	23.174
7		**AmM2**	1659.8130	1.8	353.235
8		**AmE3**	1459.8480	1.81	307.737
9		**AmEtA4**	211.0201	1.79	23.590
Compound 31	**Withaferin deriv GG-AM-4**				
1		**WsM2**	11.2940	23.07	< 0.001
Compound 32	**Eupatorin (3',5-Dihydroxy-4',6,7-trimethoxyflavone)**				
1		**ObA1**	44.5180	6.42	22.874
2		**ObM2**	217.4830	6.42	111.503
3		**ObE3**	1305.0280	6.42	668.772
Compound 33	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**				
1		**ObA1**	75.5380	7.33	38.769
2		**ObM2**	655.0720	7.32	335.728
3		**ObE3**	3619.8580	7.32	1854.916
Compound 34	**Helenin : Isoalantolactone**				
1		**IhM2**	2463.0110	8.34	1262.135
2		**IhE3**	2223.4920	8.34	1139.403
3		**IhEtA4**	2581.1450	8.34	1322.668
Compound 35	**Apigenin**				
1		**IhA1**	60.5130	6.13	31.070
2		**IhM2**	977.9820	6.13	501.190
3		**IhE3**	886.9750	6.13	454.558
4		**IhEtA4**	1041.6290	6.13	533.804
Compound 36	**Withanolide A**				
1		**WsM2**	3435.2220	5.54	1760.306
2		**WsEtA4**	3914.2630	5.54	2005.772
Compound 37	**Withaferin A**				
1		**WsM2**	1453.5440	5.92	744.873
2		**WsEtA4**	2181.1220	5.92	1117.692
Compound 38	**2,25-Diepoxywithaferin A**				
1		**WsM2**	1285.5780	5.7	658.806
2		**WsEtA4**	1439.2740	5.7	737.561
Compound 39	**Withanolide B**				
1		**WsM2**	1249.7840	7.53	640.465
		WsEtA4	1790.1690	7.53	917.364
Compound 40	**Vicosalactone B**				
1		**WsM2**	846.4430	4.71	433.788
2		**WsEtA4**	1197.2560	4.7	613.549
Compound 41	**Withanamide D/E**				
1		**WsM2**	680.3950	4.38	348.704
2		**WsEtA4**	307.8630	4.38	157.814
Compound 42	**Withasomidienone**				
1		**WsM2**	60.0101	7.22	30.812
2		**WsEtA4**	137.7530	7.22	70.648

Compound 43	**Astragaloside IV**				
1		**AmM2**	15.7301	5.60	8.122
2		**AmE3**	49.5440	5.60	25.449
3		**AmEtA4**	17.7540	5.60	9.159
Compound 44	**Astragaloside I**				
1		**AmM2**	194.4810	6.88	99.716
2		**AmE3**	923.8080	6.88	473.431
3		**AmEtA4**	718.0280	6.88	367.987
Compound 45	**Ononin**				
1		**AmM2**	138.3420	4.09	70.950
2		**AmE3**	292.6760	4.09	150.032
3		**AmEtA4**	142.2420	4.09	72.948
Compound 46	**Astragaloside II**				
1		**AmM2**	77.5830	6.05	39.816
2		**AmE3**	391.8720	6.05	200.861
3		**AmEtA4**	240.5150	6.05	123.305

***** The relatively quantified concentrations are reasonable approximations of the relative amounts of the identified compounds present in each extract (Bhandari and Rajbhandari, 2015; Punyasiri et al., 2015), ****** All the acid compounds are relatively quantified equivalent to the linear curve of gallic acid, ******* All the non- acid compounds are relatively quantified equivalent to the linear curve of quercetin.

As per the data in table 15, the concentration of salvigenin was found to be highest in ObE3 (1854.916 mg/L), along with the fatty acid trihydroxy octadecenoic acid (530.474 mg/L), eupatorin (668.772 mg/L) and caffeic acid (580.949 mg/L). Rosmarinic acid was predominant in ObA1 (2298.037 mg/L), along with tartaric acid (798.347 mg/L), isocitric (999.946 mg/L), caftaric (545.019 mg/L) and chicoric acids (496.228 mg/L).

Caffeoylquinic acid derivatives were most prominently present in IhA1 (330.846 mg/L). Epigallocatechin gallate was mostly present in IhM2 along with tanacetol A (369.886 mg/L). Macrophyllilactone B and helenin (isoalantolactone) was mostly present in IhEtA4 extract (311.470 mg/L and 1322.668 mg/L respectively).

High concentration of trihydroxy octadecenoic acid was noted in WsEtA4 (1196.370 mg/L), along with withaperuvin I (235.370 mg/L), withanolide A (2005.772 mg/L), withaferin A (1117.692 mg/L), diepoxywithaferin A (737.561 mg/L), withanolide B (917.364 mg/L) and vicosalactone B (613.549 mg/L).

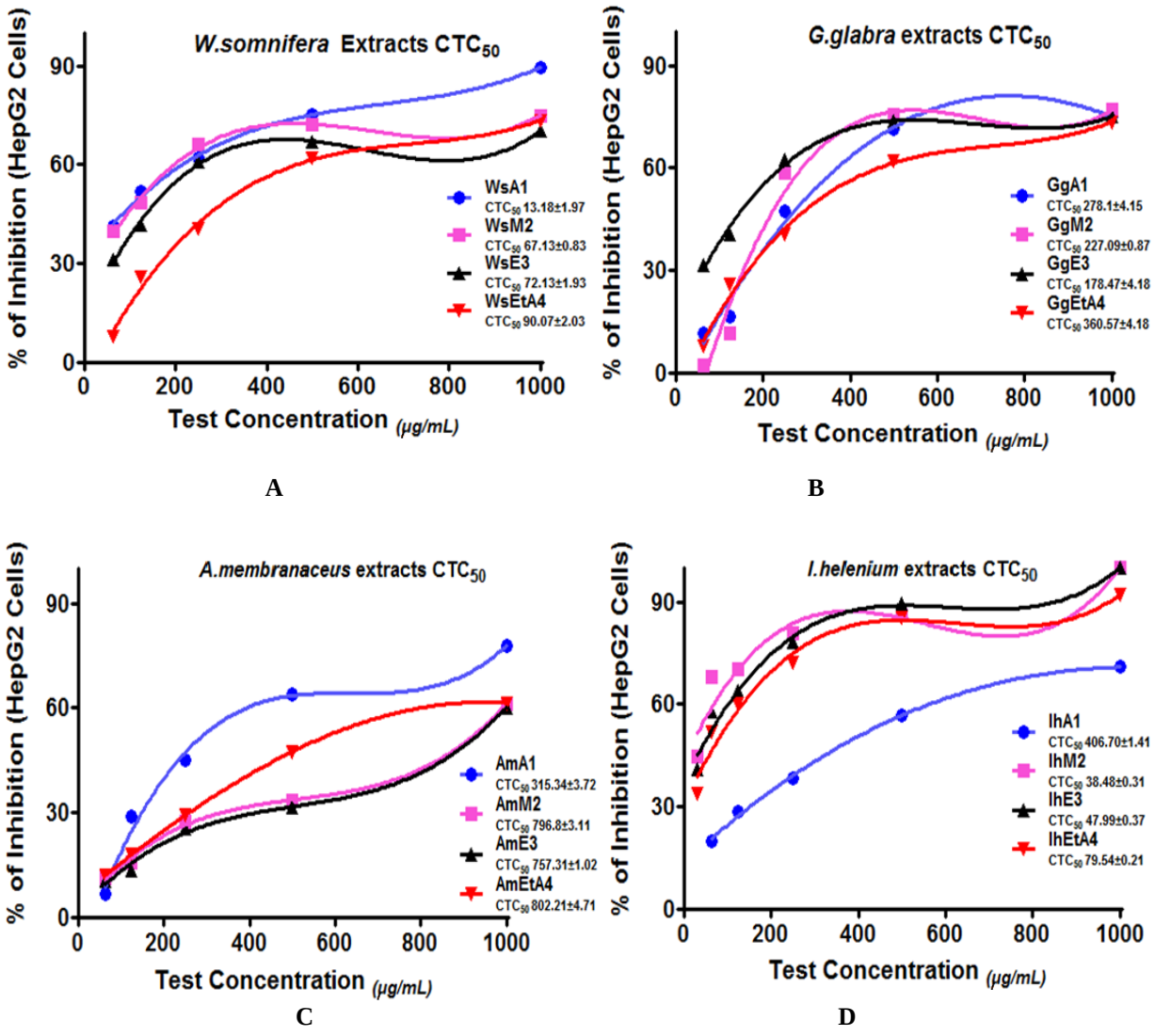
Amongst all extracts, dihexose sugar and trihydroxy octadecenoic acid was most present in *Astragalus* extracts. Calycosin and formononetin was present most in AmE3 (684.621 mg/L and 718.691 mg/L respectively). Highest concentrations of astragaloside I and II were noted in

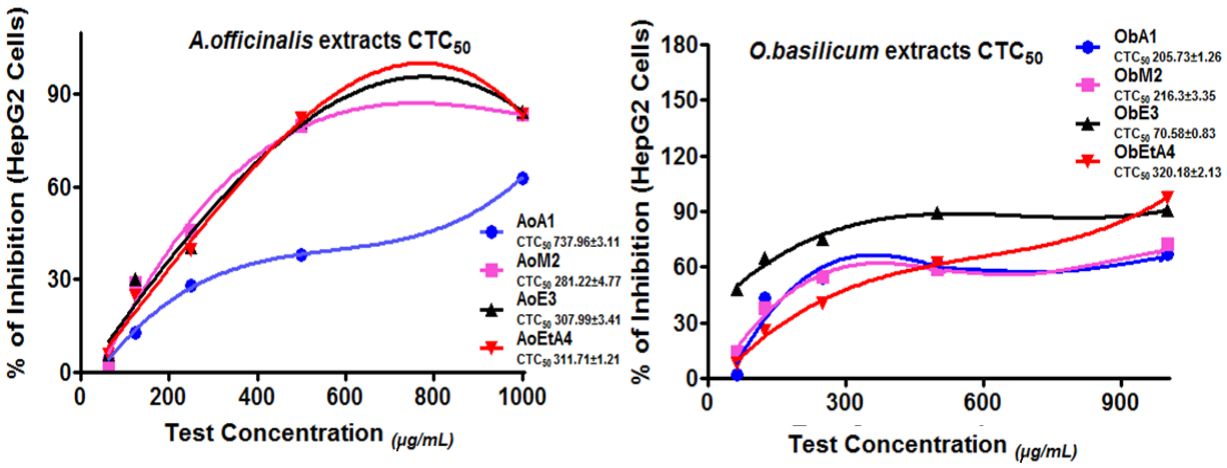
AmE3 (473.431 mg/L and 200.861 mg/L respectively). Isocitric acid was only found in AmM2 (120.228 mg/L).

3.5 Induction assays

3.5.1 Screening of herbal extracts – determination of CTC_{50}

In the MTT assays, all plant extracts were screened and tested for their *in vitro* cytotoxicity effect against HepG2 cells, by exposing the cells to different concentrations of each extract. Based on the assays the calculated CTC_{50} for all the extracts were in the range of 38-796 μg/mL for a screening concentration range between 31.25 and 1000.00 μg/mL, indicating stronger inhibition of HepG2 cell growth by various extracts at higher concentrations (Fig. 45, table 16).





E **F**

Fig. 45 Cytotoxicity % level graphs of all extracts on HepG2 cells A: *W.somnifera* B: *G.glabra* C: *A.membranaceus* D: *I.helenium* E: *A.officinalis* F: *O.basilicum*; the various test concentrations of each extract is plotted against the percentage of inhibition (dose-response) caused in the activity of the HepG2 cells.

Table [16]. Concentration of each extract that causes 50% cytotoxicity in HepG2 cells (CTC_{50}). CTC_{50} calculated as the mean ± SD of cytotoxicity % values on HepG2 cells at concentration range 1000.00-31.25 µg/mL for each extract.

Sample Name	Concentration(µg/ml)	Cytotoxicity (%)	CTC_{50} (µg/ml) #
WsA1	1000 500 250 125 62.5	89.56±0.28 75.36±0.54 62.02±0.30 52.06±0.47 41.25±0.31	**113.18±1.97**
WsM2	1000 500 250 125 62.5	74.97±0.54 72.14±0.56 66.16±0.44 48.46±0.25 39.85±0.31	**67.13±0.83**
WsE3	1000 500 250 125 62.5	70.21±0.54 67.01±0.56 61.09±0.44 41.72±1.56 31.05±0.04	**72.13±1.93**
WsEtA4	1000 500 250 125 62.5	73.43±0.57 61.92±0.54 40.53±0.87 25.77±0.54 7.85±0.45	**90.07±2.03**
GgA1	1000 500 250 125 62.5	75.10±0.45 71.30±0.22 47.30±0.43 16.60±1.09 11.70±0.97	**278.1±4.15**
GgM2	1000 500 250 125 62.5	76.96±0.45 75.45±0.66 58.60±0.33 11.66±0.65 2.38±0.78	**227.09±0.87**

GgE3	1000 500 250 125 62.5	75.16±0.22 73.58±0.33 62.42±1.51 40.75±0.76 31.53±1.42	**178.47±4.18**
GgEtA4	1000 500 250 125 62.5	73.43±0.57 61.92±0.54 40.53±0.87 25.77±0.54 7.85±0.45	**360.57±4.18**
AmA1	1000 500 250 125 62.5	77.80±0.34 64.03±0.34 45.04±0.26 28.80±1.05 6.60±0.98	**315.34±3.72**
AmM2	1000 500 250 125 62.5	61.30±0.19 33.50±0.19 27.40±0.11 15.70±0.24 11.40±0.57	**796.80±3.11**
AmE3	1000 500 250 125 62.5	60.30±1.34 31.50±0.34 25.40±2.26 13.70±1.05 10.40±0.71	**757.31±1.02**
AmEtA4	1000 500 250 125 62.5	61.45±0.49 47.52±0.89 29.31±3.11 18.01±0.24 12.01±0.17	**802.21±4.71**
IhA1	1000 500 250 125 62.5	70.91±0.38 56.95±0.30 38.33±0.23 28.61±0.20 19.75±2.75	**406.70±1.41**
IhM2	1000 500 250 125 62.5 31.25	99.91±3.96 86.05±0.56 80.92±0.72 70.15±0.33 67.92±0.97 44.61±0.31	**38.48±0.31**
IhE3	1000 500 250 125 62.5 31.25	99.87±1.99 89.46±0.41 78.01±0.54 64.07±0.41 57.95±0.37 40.83±0.30	**47.99±0.37**
IhEtA4	1000 500 250 125 62.5 31.25	92.01±1.07 85.46±1.41 72.01±2.59 60.07±0.41 51.95±1.37 33.83±0.09	**79.54±0.21**
AoA1	1000 500 250 125 62.5	63.05±0.55 38.15±0.26 27.94±0.60 12.85±0.78 4.70±0.81	**737.96±3.11**

AoM2	1000 500 250 125 62.5	83.42±0.36 79.92±0.36 45.73±0.70 28.86±0.65 3.04±0.26	**281.22±4.77**
AoE3	1000 500 250 125 62.5	84.45±0.87 81.24±0.46 40.56±0.60 30.12±0.86 5.62±1.21	**307.99±3.41**
AoEtA4	1000 500 250 125 62.5	83.45±1.87 82.24±1.46 39.56±0.20 25.01±0.86 5.67±2.21	**311.71±1.21**
ObA1	1000 500 250 125 62.5	66.95±1.35 61.34±1.73 53.85±0.54 42.98±0.94 2.23±0.33	**205.73±1.26**
ObM2	1000 500 250 125 62.5	72.20±1.17 58.80±0.78 54.50±0.72 37.90±0.52 14.10±1.05	**216.30±3.35**
ObE3	1000 500 250 125 62.5	90.75±0.15 89.13±0.67 75.38±0.54 65.08±0.33 47.75±0.31	**70.58±0.83**
ObEtA4	1000 500 250 125 62.5	97.43±0.15 61.92±1.67 40.53±2.54 25.77±0.33 7.86±0.31	**320.18±2.13**
[#] Values are represented as mean ± SD (n = 3).			

As shown in table 16, IhM2 showed the lowest CTC_{50} value of 38.48±0.31 μg/mL, compared to all other extracts which indicated its potent toxicity levels on the HepG2 cells at higher concentrations. AmM2 had the highest CTC_{50} value of 796.8±3.11 μg/mL, which indicated that it was much less cytotoxic at higher concentrations. All extracts of *W.somnifera* showed CTC_{50} values in the range of 67-113 μg/mL, the aqueous extract having the highest value. All the extracts of *A.membranaceus*, had CTC_{50} values > 315 μg/mL.

3.5.2 mRNA expression – using CTC_{50} concentration of herbal extracts

To analyse the inducing effect of the herbal extracts on the mRNA expression of HepG2 cells, using RT-PCR technique, they were screened using their CTC_{50} concentrations (as shown in section 3.5.1). Relative mRNA expressions of CYP3A4 by the herbal extracts on agarose gel are represented in figure 46. The expression levels of CYP2B6 and CYP3A4 are depicted as arbitrary units normalized to (GAPDH) mRNA.

For the induction screening against CYP2B6, dexamethasone (DEX, 10µM) was used as the positive control, and showed significant fold induction ($p < 0.001$) compared to the cell control (CC).Compared to DEX, none of the extracts showed 2-fold induction (200% increase in fold-shift) and most of them were only moderate inducers of CYP2B6 (< 50%-fold shift). ObM2 induced CYP2B6 mRNA the most; followed by AmA1 with 36%-fold response increase compared to CC. The methanolic and ethanolic extracts of *O.basilicum* and *G.glabra* moderately induced CYP2B6 mRNA with 38%- and 28%-fold shift and 26%- and 33%-fold shift respectively, compared to CC. All the extracts of *W.somnifera* showed least induction of CYP2B6 mRNA, with fold responses < 20%, compared to CC (fig 47).

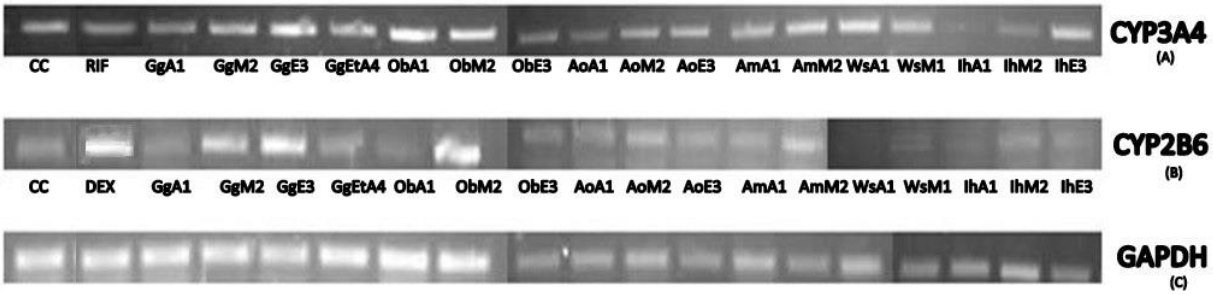


Fig.46 Relative (A) CYP3A4 and (B) CYP2B6 mRNA expressions by CC, RIF, DEX and the extracts on agarose gel. (C) GAPDH mRNA expression on agarose gel relative to CC, RIF, DEX and the extracts for normalization.

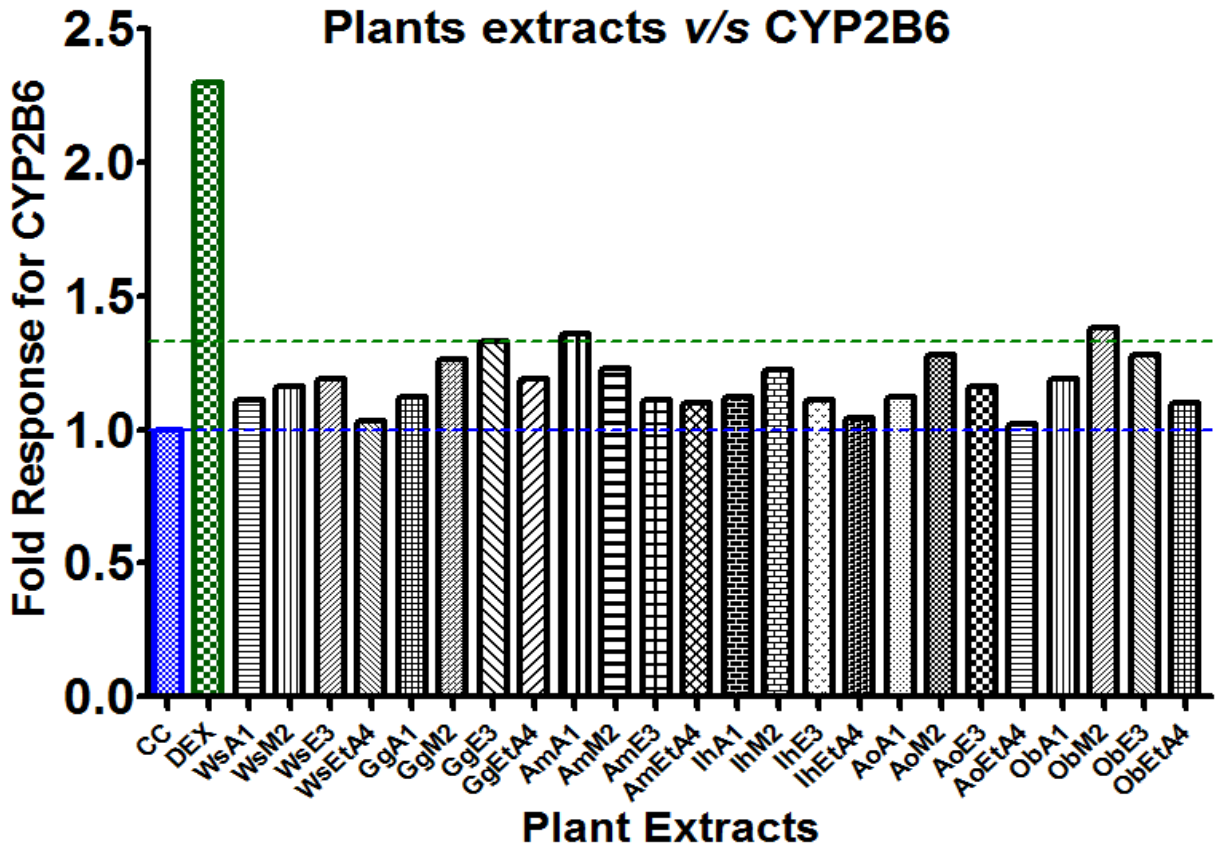


Fig.47 Histograms of all the herbal extracts and their fold responses for CYP2B6 mRNA expression, relative to cell control (CC); DEX is the positive control.

For CYP3A4, most of the extracts showed inducing potential. The positive control rifampicin (RIF, 50 µM) showed significant fold induction ($p < 0.001$) compared to the cell control (CC). When compared to RIF, none of the extracts showed 2-fold induction of CYP3A4. However on an average scale compared to CC, most of the extracts moderately induced CYP3A4 mRNA, with an average fold response > 25% of the CC (fig 48).

Relative to CC, the ethanolic and methanolic extracts of *A.officinalis* induced CYP3A4, with 48%- and 44%-fold increases in gene expression. AoE3 induced the most with 48%-fold response (0.5-fold induction). The extracts of *O.basilicum* induced 3A4, with ObM2 showing 22%-fold response increase and ObE3 at 44%. All the extracts of *A.membranaceus* showed good induction of CYP3A4, with an average fold response of 37% compared to CC (fig 48).

For *W.somnifera* extracts, WsA1 induced CYP3A4 with 38%-fold shift followed by WsM2 and WsM3 (30%increase in the fold response). Comparatively, all extracts of *G.glabra* showed less induction with average fold response < 15%.

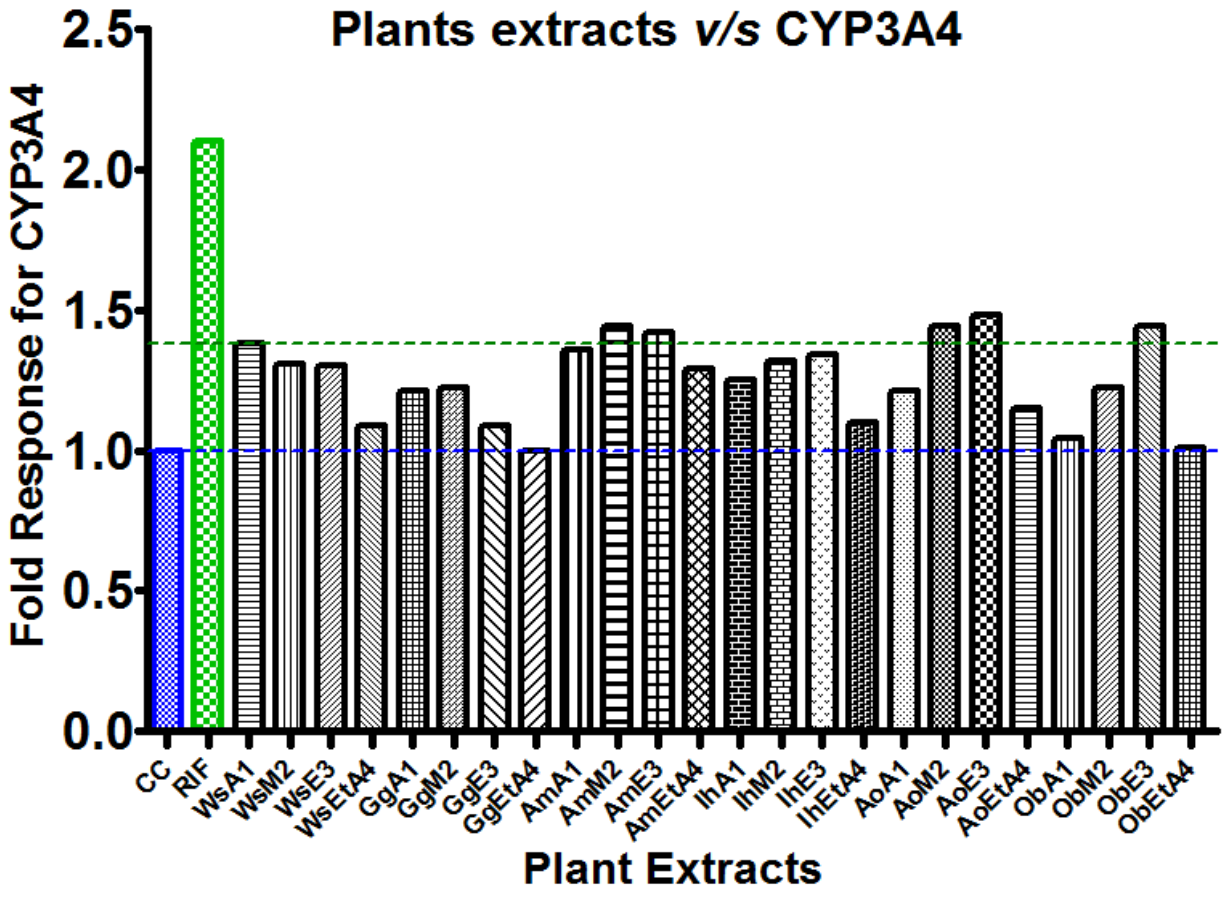


Fig.48 Histograms of all the herbal extracts and their fold responses for CYP3A4 mRNA expression, relative to cell control (CC); DEX is the positive control.

The aqueous extract of *A.membranaceus* consistently induced both CYP2B6 and CYP3A4 mRNA expression with 36%-fold increase in the gene expression (fig.47, 48) indicating that this extract is a moderate inducer of the CYPs. However none of the extracts induced both CYPs over 2-fold, indicating that the phytoconstituents present in these extracts are not strong inducers of the CYPs. The mRNA expression assays depicted that CYP3A4 was more inducible by the investigated herbs compared to CYP2B6.

3.6 Phytochemical fingerprinting – Extracts inducing CYP activity

3.6.1 LC-MS/ PDA analysis

For the negative mode extract scans, the following observations were noted (table 17):

1. *Althaea officinalis* (methanol and ethanol) extracts (fig. 49 shows the chromatogram of the ethanol extract):
    - The most prominent peak in both the extracts of *A.officinalis* was tentatively identified as quinic acid derivative [quinic acid−H−H2O]$^-$ (fig. 50 a) (Li et al., 2016; Chang et al., 2016) with a retention time of 17.16 min at *m/z* 173 at a PDA detection wavelength 230 nm and daughter ions at *m/z* 83, 111, 127 and 173.

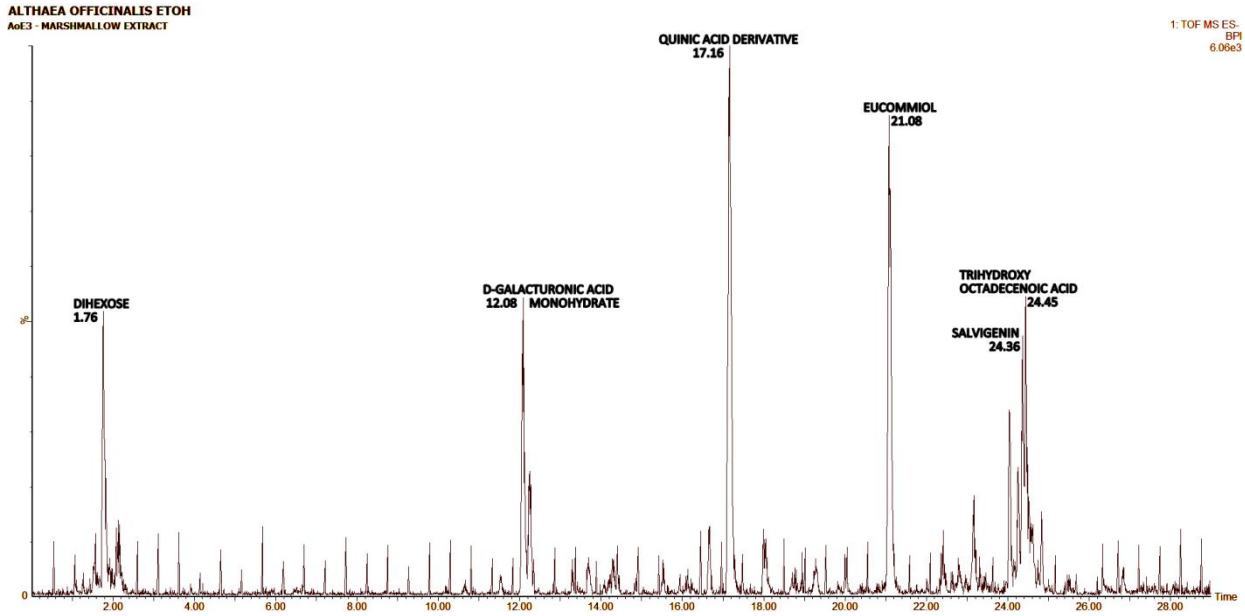


Fig. 49 LC-MS chromatogram of *A.officinalis* (ethanol extract) in negative mode scan for time duration of 29 min.

- Another prominent peak observed in both extracts was identified as eucommiol, at retention time 21.09 min at *m/z*187 and fragment ions at *m/z* 125, 141, 169, and 187.
- D-Galacturonic acid monohydrate was observed in the ethanolic extract at *m/z* 211 and retention time 12.08 min (fig. 50 b).

- The fatty acid trihydroxy-octadecenoic acid (329 *m/z*) was prominently observed in the ethanolic extract.
- Other common compounds included salvigenin (327 *m/z*) and dihexose sugar (341 *m/z*).

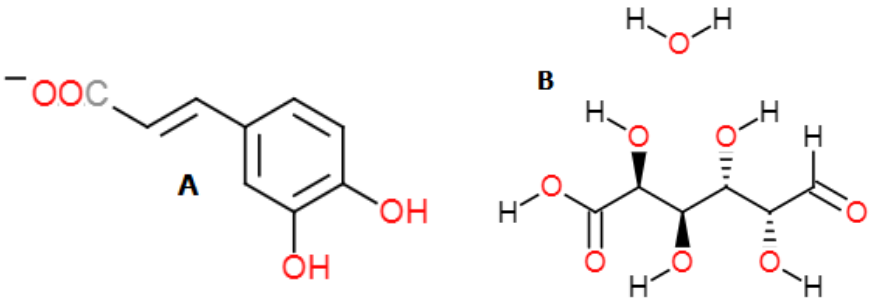


Fig.50 Structures of some of the main compounds identified from crude herbal extracts of *A.officinalis*: (A) Quinic acid derivative; (B) D-Galacturonic acid, monohydrate.

2. *Glycyrrhiza glabra* (ethanol) extract (fig. 51 shows the chromatogram of the ethanol extract):
   - The most prominent peak identified as the known chalcone, licochalcone B observed at a retention time of 23.91 min (fig. 51 a) with *m/z* 285 at a PDA detection wavelength 230 nm and fragment ions at *m/z* 150, 270 and 285 (table 17).

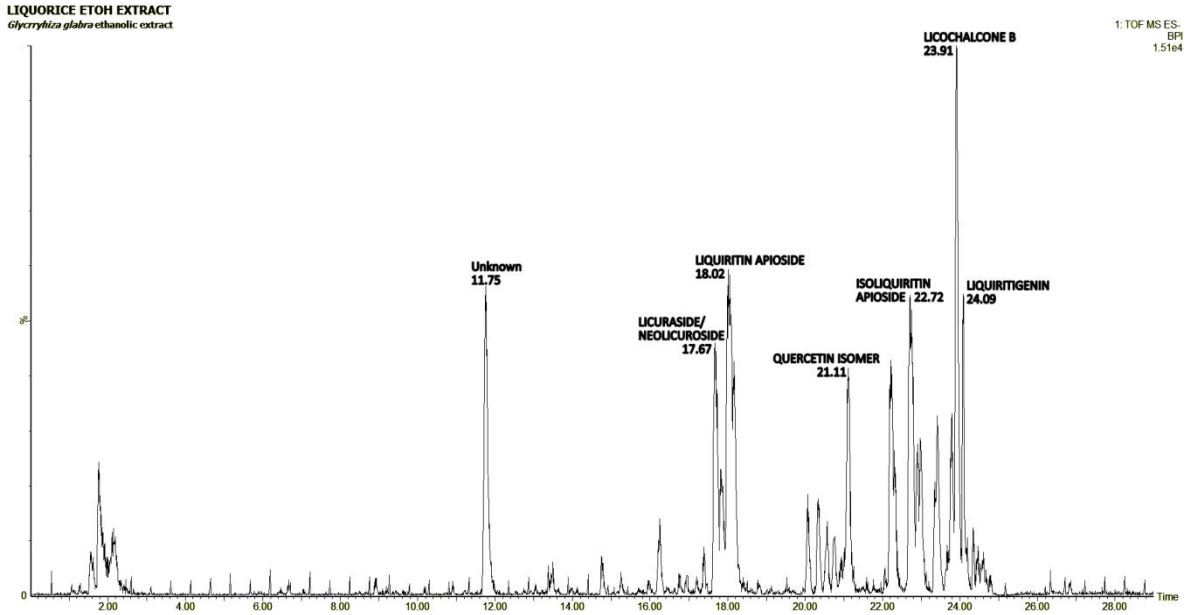


Fig. 51 LC-MS chromatogram of *Glycyrrhiza glabra* (ethanol extract) in negative mode scan for time duration of 29 min.

- The second most prominent peak observed was identified as a flavonone, liquiritin apioside at retention time 18.02 min, *m/z* 549 and daughter ions at *m/z* 255, 285 and 429 (fig. 51 b).

- Other known compounds identified included liquiritigenin (255 *m/z*) and licuraside/ neolicuroside (549 *m/z*).

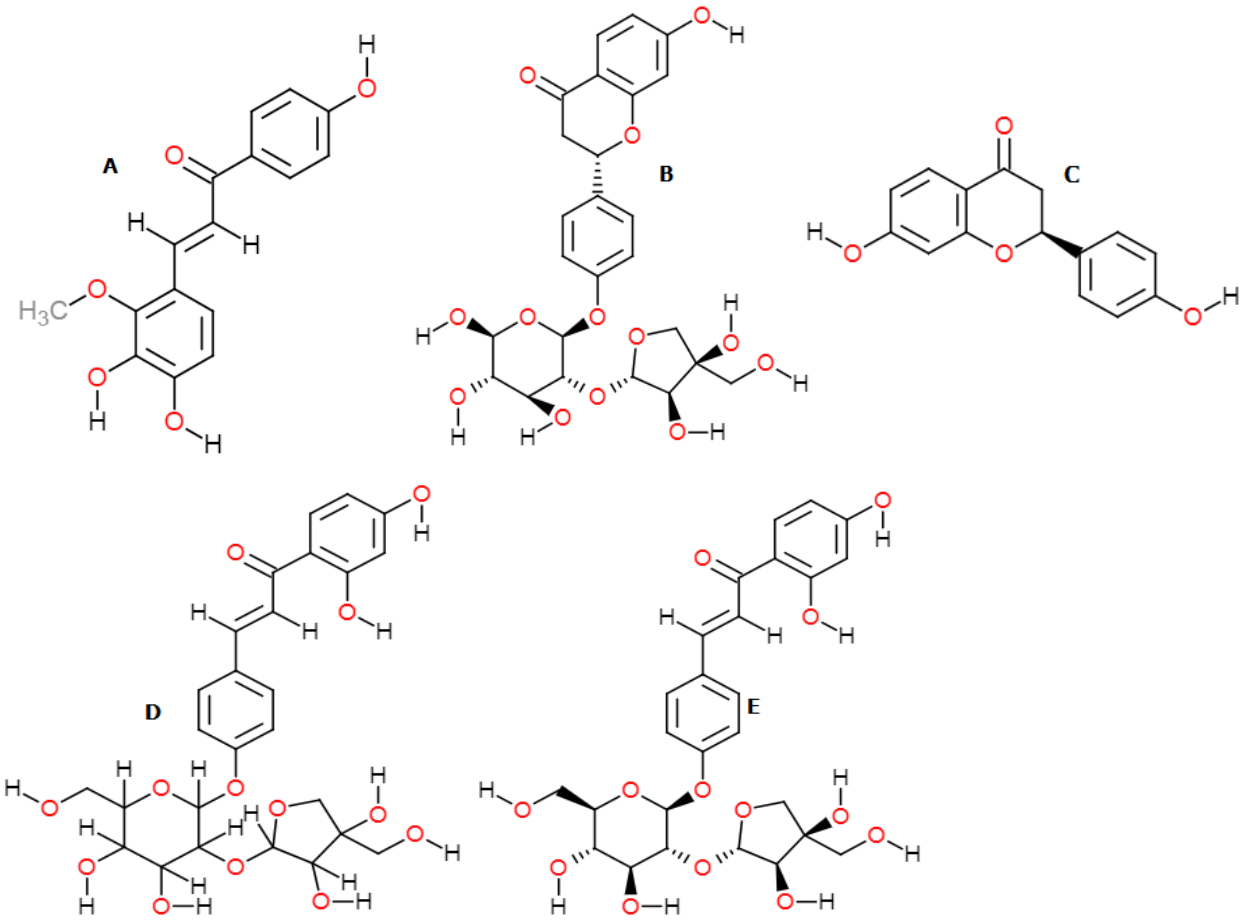


Fig.52 Structures of some of the main compounds identified from crude herbal extracts of *Glycyrrhiza glabra*: (A) Licochalcone B; (B) Liquiritin apioside; (C) Liquiritigenin; (D) Licuraside; (E) Neolicuroside.

3. *Astragalus membranaceus* (aqueous) extract:

- The phytochemical profile of the aqueous extract was very similar to the organic solvent extracts of *A.membranaceus*. The major compounds observed included isocitric acid at 3.72 min (191 *m/z*), dihexose sugar (341 *m/z*) at 1.76 min, trifolirhizin (491 *m/z*) at 18.01 min, quercetin isomer (301 *m/z*) at 21.11 min, and calycosin (283 *m/z*) and formononetin (267 *m/z*) at 24.21 and 24.56 min respectively (table 17).

Table [17] Compounds detected in *A.officinalis*, *G.glabra* and *A.membranaceus* extracts in negative scan mode in LC-MS/ PDA.

SL NO.	RT (MIN)	M-H	$[M-H]^-$	MS/MS[C]	TENTATIVE ID[#]	COMPOUND NATURE
AoMeOH						
1	17.14	173.0802	$C_8H_{13}O_4$	83, 111, 127	**Quinic Acid derivative [quinic acid−H−H2O]$^-$**	Carboxylic acid
2	21.09	187.0978	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cylopentenoid-tetrol
3	24.36	327.2158	$C_{18}H_{31}O_5$	116.9, 205, 215, 277, 311,	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavones

4	1.76	683.2255	$C_{25}H_{13}N_2$	377, 503	***Unknown***	–
5	1.80	341.1066	$C_{12}H_{21}O_{11}$	89, 119, 179, 341	**Dihexose**	Sugar
6	24.03	312.1241	$C_{19}H_{14}N_5$, $C_{18}H_{18}NO_4$	148, 178, 312	***Unknown***	–
7	16.22	358.0936	$C_{18}H_{16}NO_7$, $C_{19}H_{12}N_5O_3$	135,160, 178, 222, 358	***Unknown***	–
AoEtOH						
1	17.16	173.0813	$C_8H_{13}O_4$	83, 111, 127	**Quinic Acid derivative [quinic acid−H−H2O]$^-$**	Carboxylic acid
2	21.08	187.0968	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cylopentenoid-tetrol
3	24.45	329.2330	$C_{18}H_{33}O_5$	116.92,171, 211, 258	**Trihydroxy-octadecenoic acid**	Fatty acid
4	12.08	211.0640	$C_6H_{11}O_8$	96, 127, 162	**D-Galacturonic acid, monohydrate**	Sugar acid
5	1.76	341.1066	$C_{12}H_{21}O_{11}$	89, 119, 179	**Dihexose**	Sugar
6	24.36	327.2175	$C_{18}H_{15}O_6$	116.9, 205, 215, 277, 311	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**	Flavones
7	24.04	312.1232	$C_{18}H_{18}NO_4$	148, 178	***Unknown***	–
GgEtOH						
1	23.91	285.0758	$C_{16}H_{13}O_5$	150, 270	**Licochalcone B**	Chalcones
2	18.02	549.1607	$C_{36}H_{29}O_{13}$	255, 285, 429	**Liquiritin apioside**	Flavonones
3	11.75	209.0443	$C_{10}H_9O_5$	165	***Unknown***	–
4	24.09	255.0661	$C_{15}H_{11}O_4$	119, 135	**Liquiritigenin**	Flavonones
5	22.72	549.1612	$C_{26}H_{29}O_{13}$	255, 429	**Isoliquiritin apioside**	Chalcones
6	17.67	549.1602	$C_{26}H_{29}O_{13}$	135, 255, 297, 429	**Licuraside/ Neolicuroside**	Chalcones
7	21.11	301.0726	$C_9H_{17}O_{11}$	151, 191, 286,	**Quercetin isomer**	Flavonoid
8	1.77	341.1091	$C_{12}H_{21}O_{11}$	89, 119, 179	**Dihexose**	Sugar
AmAq						
1	3.72	191.0189	$C_6H_7O_7$	111, 129, 173	**Isocitric acid**	Citric acid
2	1.76	341.1091	$C_{12}H_{21}O_{11}$	89, 119, 179	**Dihexose**	Sugar
3	24.21	283.0612	$C_{16}H_{11}O_5$	136, 196, 211, 240, 269	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**	Isoflavone
4	10.73	205.0723	$C_8H_{13}O_6$	115,129	***Unknown***	–
5	18.01	491.1184	$C_{22}H_{22}O_{10}$ [M+FA-H]	233, 255, 268, 283	**(-)-Maackiain-3-Oglucoside (Trifolirhizin)**	Flavonoid
6	21.04	187.0945	$C_9H_{15}O_4$	125, 141, 169	**Eucommiol**	Cylopentenoid-tetrol
7	24.56	267.0654	$C_{16}H_{11}O_4$	115,117,196, 207, 236	**Formononetin (7-Hydroxy-4'-methoxyisoflavone)**	Isoflavone
8	22.98	475.1259	$C_{16}H_{27}O_{16}$	25, 267, 268	***Unknown***	–

[#]**REFERENCES** –Chen et al., 2017; Said et al., 2017; Chang et al., 2016.; Chen et al., 2016; Deters et al., 2016; Li et al., 2016; Liu et al., 2016; Soleimany et al., 2016; Zhang et al., 2016; Simirgiotis et al., 2015; Du et al., 2014; Kuo et al., 2014; Zhang et al., 2013; Mena et al., 2012; Wang et al., 2012; Farag et al., 2012; Čejchanová, 2011; Hossain et al., 2010; Li et al., 2007.
https://www.ncbi.nlm.nih.gov/pccompound; https://massbank.eu/MassBank/; https://metlin.scripps.edu/; https://webbook.nist.gov/chemistry/mw-ser/

For the positive mode extract scans, the following observations were noted (details summarized in table 18):

1. *Althaea officinalis* (methanol and ethanol) extracts (fig. 53 illustrates the chromatogram of the methanolic extract):
    - The most prominent peaks in both the extracts of *A.officinalis* were having *m/z* 398 with a retention times of 7.6 and 7.43 min at UV detection wavelength of 230 nm. These molecules resembled characteristics of the previously reported compound

altheahexacosanyl lactone with *m/z* = 391, isolated from the seeds of *A.officinalis* (Rani et al., 2010) and were tentatively identified as derivatives 1 and 2 (table 18, fig54 a).

- Some of the other peaks in both the extracts at 5.76 min (348 *m/z*), 7.86 min (412 *m/z*) and 7.91 min (412 *m/z*) could not be identified and were derived as nitrocompounds ($[M+H]^+$ formula details in table 18).
- The dihydrochalcone phloretin (fig. 54 b) was identified at 6.27 min retention time with *m/z* 275, and fragment ions at *m/z* 215 and 229.

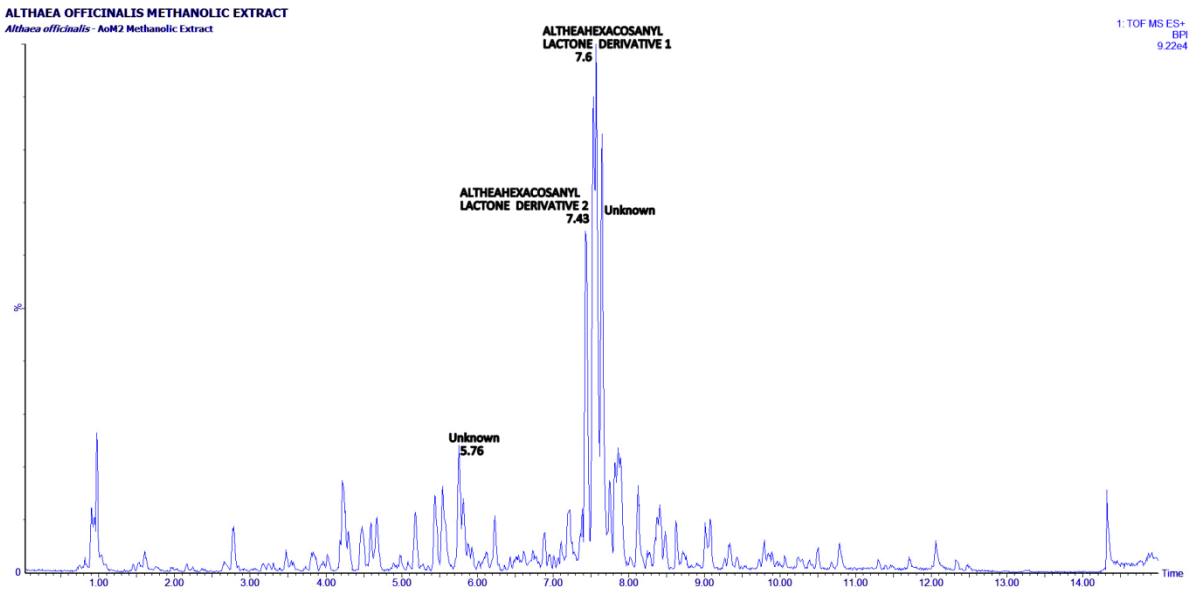


Fig. 53 LC-MS chromatogram of *Althaea officinalis* (methanol extract) in positive mode scan for time duration of 15 min.

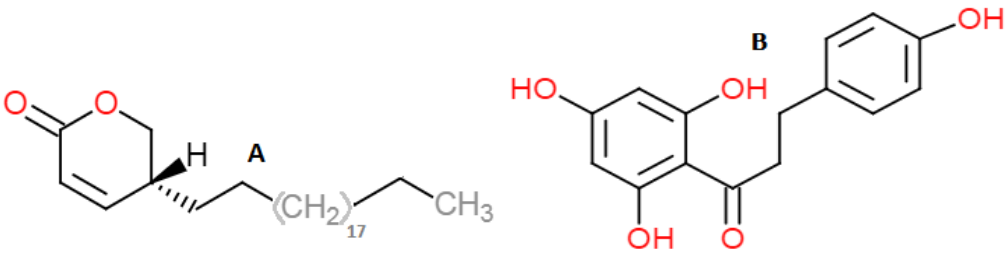


Fig. 54 Structures of some of the main compounds identified from crude herbal extracts of *Althaea officinalis* (positive mode): (A) Altheahexacosanyl lactone; (B) Phloretin.

2. *Glycyrrhiza glabra* (ethanol) extract (fig. 55 shows the chromatogram of the ethanolic extract):
   - The positive scan of the ethanol extract resulted in the identification of three major compounds in *G.glabra* namely, licochalcone C (339 *m/z*), glabrene (323 *m/z*) and glabridin (325 *m/z*) at retention time points 8.42, 7.58 and 8.86 min, respectively. The UV detection wavelength for licochalcone B was at 354 nm (fig. 56). The daughter ions

for licochalcone C where identified as *m/z* 229 and 297, whereas the products ions for glabrene and glabridin were identified as *m/z* 267 and *m/z* 123, 149, 189 respectively.

- Licoflavone B was detected at a retention time 9.8 min and *m/z* 391 at UV wavelength of 300 nm.

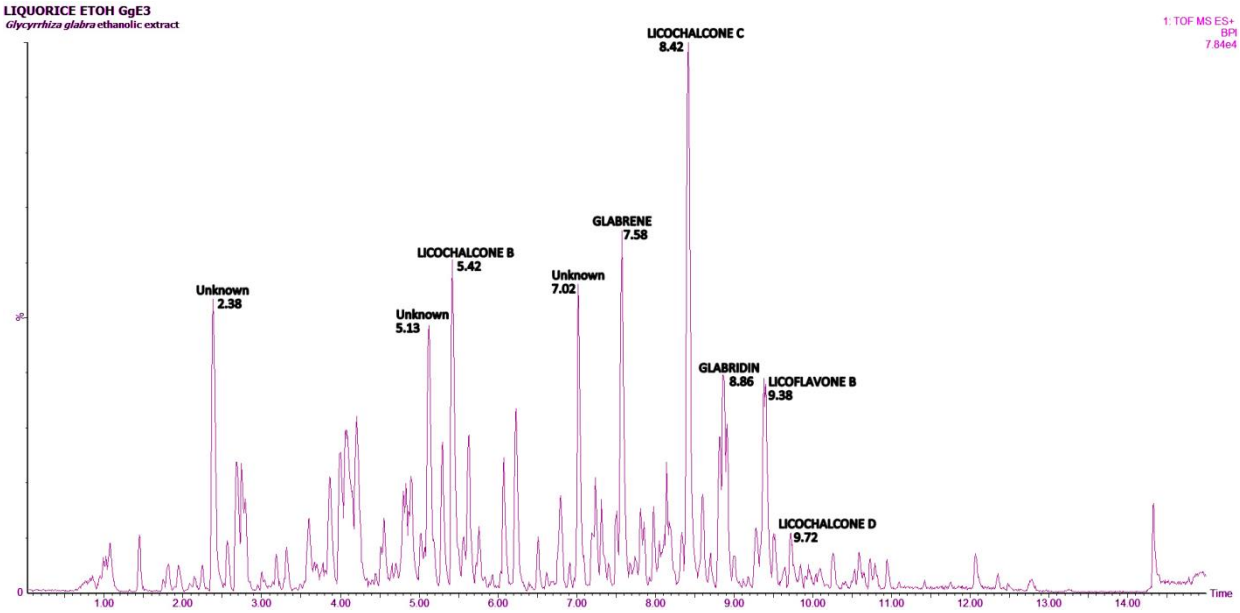


Fig. 55 LC-MS chromatogram of *Glycyrrhiza glabra* (ethanol extract) in positive mode scan for time duration of 15 min.

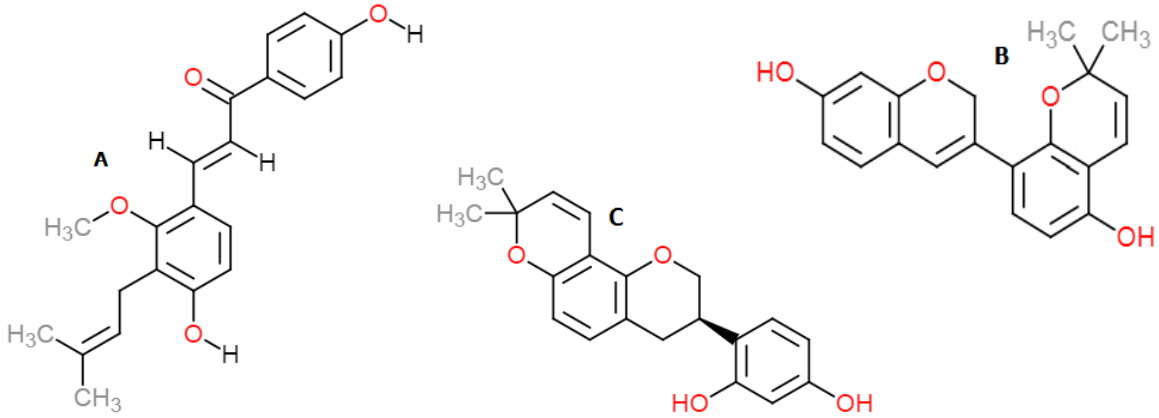


Fig.56 Structures of some of the main compounds identified from crude herbal extracts of *Glycyrrhiza glabra* (positive mode): (A) Licochalcone C; (B) Glabrene; (C) Glabridin.

3. *Astragalus membranaceus* (aqueous) extract:

- The prominent peaks were identified as physcion (285 *m/z*), physcion 8-glucoside (447 *m/z*) and apigenin 7-glucuronide (269 *m/z*) (fig. 57). Physcion had a retention time of 5.76 min and its product ions were identified as *m/z* 206, 219, 240, 268 and 270.

Physcion 8-glucoside eluted at 4.09 min, while apigenin 7-glucuronide eluted at 7.03 min.

- The bioactive saponin cycloastragenol was identified at 4.99 min, though in lesser concentrations, with *m/z* 491 and product ions *m/z* 270, 286 and 383.

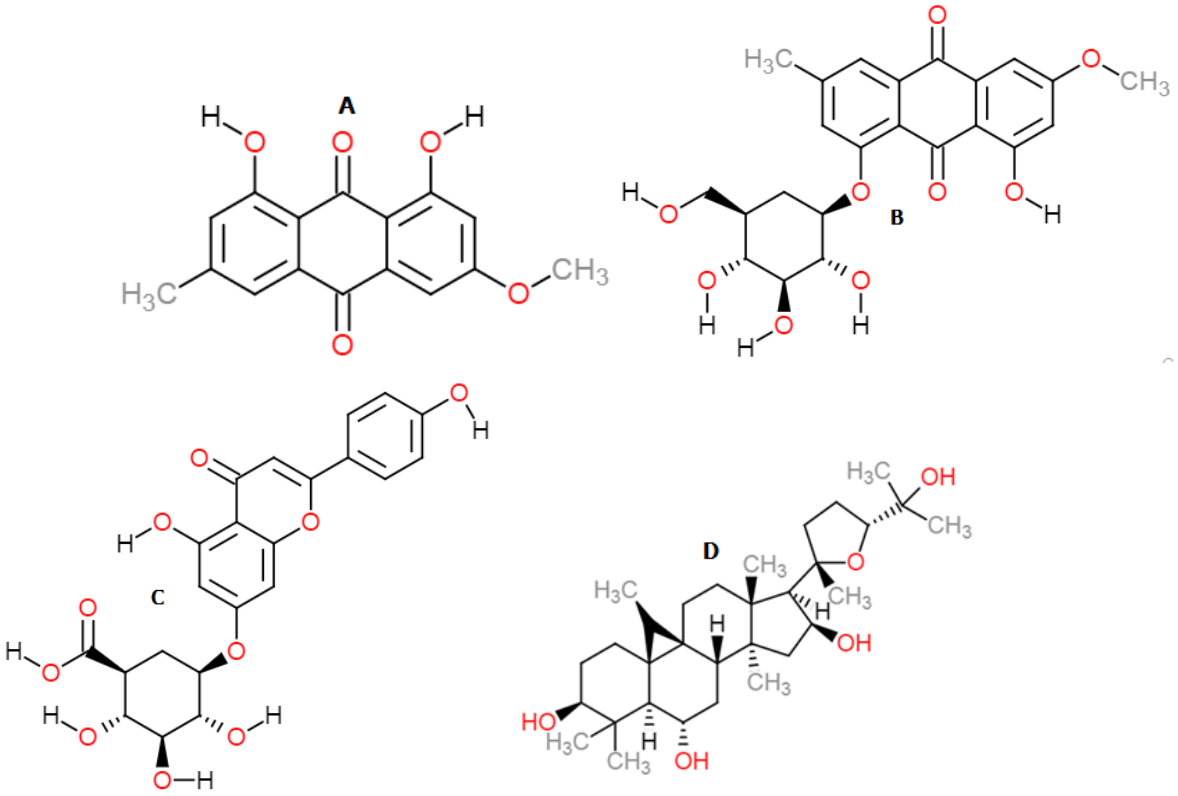


Fig.57 Structures of some of the known and proposed compounds identified from crude herbal extracts of *Astragalus membranaceus* (positive mode): (A) Physcione; (B) Physcione 8-glucoside; (C) Apigenin 7-glucuronide; (D) Cycloastragenol.

Table [18] Compounds detected in *A.officinalis*, *G.glabra* and *A.membranaceus* extracts in positive scan mode in LC-MS/PDA.

SL NO.	RT (MIN)	M+H	$[M+H]^+$	MS/MS[C]	TENTATIVE ID[#]	COMPOUND NATURE
AoM2						
1	7.60	398.3265	$C_{26}H_{54}O_2$	118, 119, 202, 231, 270, 321, 349	**Altheahexacosanyl lactone derivative 1?**	Lactone
2	7.43	398.3266	$C_{26}H54O_2$	118, 136, 331	**Altheahexacosanyl lactone derivative 2?**	Lactone
3	5.76	348.2172	$C_{20}H_{30}NO_4$	128, 219, 302	***Unknown***	–
4	7.86	412.3422	$C_{24}H_{46}NO_4$	118, 301, 332	***Unknown***	–
AoE3						
1	7.65	398.3267	$C_{26}H_{54}O_2$	118, 119, 202, 231, 270, 321, 349	**Altheahexacosanyl lactone derivative 1?**	Lactone
2	7.44	398.3258	$C_{26}H_{54}O_2$	118, 136, 331	**Altheahexacosanyl lactone derivative 2?**	Lactone
3	7.91	412.3416	$C_{24}H_{46}NO_4$	118, 184, 333	***Unknown***	–
4	6.27	275.0906	$C_{15}H_{15}O_5$	215, 229	**Phloretin**	Dihydrochalcone
GgE3						
1	8.42	339.1591	$C_{21}H_{23}O_4$	229, 297	**Licochalcone C**	Chalcone
2	7.58	323.1274	$C_{20}H_{19}O_4$	267	**Glabrene**	Isoflavonoid

3	5.42	287.0912	$C_{16}H_{15}O_5$	121, 240	**Licochalcone B**	Chalcone
4	7.02	269.0807	$C_{16}H_{13}O_4$	197, 253	***Unknown***	–
5	5.13	431.1343	$C_{22}H_{23}O_9$	269	***Unknown***	–
6	8.86	325.1439	$C_{20}H_{21}O_4$	123, 149, 189	**Glabridin**	Isoflavane
7	9.38	391.1907	$C_{25}H_{25}O_4$	333	**Licoflavone B**	Flavones
8	9.72	355.0125	$C_{21}H_{23}O_5$	297, 338	**Licochalcone D**	Chalcones
AmA1						
1	5.76	285.0763	$C_{16}H_{13}O_5$	206, 219, 240, 268, 270	**Physcion**	Flavonoid
2	14.32	247.1665	$C_{11}H_{23}N_2O_4$, $C_{16}H_{23}O_2$	124, 148, 226	***Unknown***	–
3	4.09	447.1295	$C_{22}H_{23}O_{10}$	269, 270, 285	**Physcion 8-glucoside**	Anthraquinone glucoside
4	7.22	192.1388	$C_{12}H_{18}NO$	91, 119, 167	***Unknown***	–
5	7.03	269.0805	$C_{16}H_{13}O_4$	197, 225, 237	**Apigenin 7-glucuronide**	Flavone metabolite
6	1.65	259.0928	$C_{10}H_{14}N_3O_5$, $C_8H_{18}NO_8$	150, 196, 210	***Unknown***	–
7	5.12	431.1347	$C_{22}H_{23}O_9$	269, 270	***Unknown***	–
8	4.99	491.1021	$C_{30}H_{49}O_5$	270, 286, 383	**Cycloastragenol**	Bioactive Saponin

[#]**REFERENCES** – Sendker et al., 2017; Wintola and Afolayan, 2017; Deters et al., 2016; Soleimany et al., 2016; Liu et al., 2016; Du et al., 2014; Bolleddula et al., 2012; Wang et al., 2012; Farag et al., 2012; Čejchanová, 2011; Hossain et al., 2010; Rani et al., 2010; Li et al., 2007, Ye et al., 2005. https://www.ncbi.nlm.nih.gov/pccompound; https://massbank.eu/MassBank/; https://metlin.scripps.edu/; https://webbook.nist.gov/chemistry/mw-ser/

The major identified compounds for the three active extracts were relatively quantified (in mg/L equivalent units of gallic acid/ quercetin), with respect to the linear calibration curve of the reference standards quercetin and gallic acid (table 19). The major compounds altheahexacosanyl lactone derivatives 1 & 2, were relatively quantified at 3874.310 and 1341.994 mg/L in AoM2 and 4324.664 and 1903.778 mg/L in AoE3, respectively. In GgE3 extract, licochalcone C was the most prominent compound (2208.600 mg/L) followed by glabrene and licochalcone B. For AmA1, all the identified compounds were found to be < 500 mg/L in concentration.

Table [19] Relative quantification of the major compounds identified in *A.officinalis*, *G.glabra* and *A.membranaceus* extracts in positive scan mode in LC-MS/ PDA.

# SLNO	COMPOUND NAME	SAMPLE NAME	AREA	RT(MIN)	CONC. UNITS* (MG/L EQUIVALENTS OF GALLIC ACID/ QUERCETIN)
Compound 1	**Altheahexacosanyl lactone derivative 1**				***
1		**AoM2**	7560.8280	7.60	3874.310
2		**AoE3**	8439.7201	7.65	4324.664
Compound 2	**Altheahexacosanyl lactone derivative 2**				
1		**AoM2**	2618.8600	7.43	1341.994
2		**AoE3**	3715.2160	7.44	1903.778
Compound 3	**Phloretin**				
1		**AoE3**	2103.2310	6.27	1077.780
Compound 4	**Salvigenin (5-Hydroxy-6,7,4'-trimethoxyflavone)**				

1		**AoM2**	190.1650	24.36	18.845
2		**AoE3**	154.5610	24.36	10.744
Compound 5	**Trihydroxy octadecenoic acid**				****
1		**AoE3**	189.0380	24.45	249.011
Compound 6	**Eucommiol**				
1		**AoM2**	365.7730	21.09	58.802
2		**AoE3**	321.1200	21.08	48.642
Compound 7	**Dihexose**				
1		**AoM2**	117.9770	1.80	2.420
2		**AoE3**	168.5270	1.76	13.922
3		**AmA1**	131.4520	1.76	5.486
Compound 8	**Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone)**				
1		**AmA1**	105.4160	24.21	0.070
Compound 9	**Isocitric acid**				
1		**AmA1**	174.1490	3.72	228.809
Compound 10	**Apigenin 7-glucuronide**				
1		**AmA1**	472.3550	7.03	242.102
Compound 11	**Licochalcone C**				
1		**GgE3**	4310.0950	8.42	2208.600
Compound 12	**Glabrene**				
1		**GgE3**	2808.0590	7.58	1438.941
Compound 13	**Licochalcone B**				
1		**GgE3**	2377.3450	5.42	1218.239
Compound 14	**Glabridin**				
1		**GgE3**	1454.4840	8.86	745.355
Compound 15	**Licoflavone B**				
1		**GgE3**	1083.8200	9.38	555.423
Compound 16	**Licochalcone D**				
1		**GgE3**	387.8520	9.72	198.802
Compound 17	**Physcion**				
1		**AmA1**	839.8840	5.76	430.428
Compound 18	**Physcion 8-glucoside**				
1		**AmA1**	349.3340	4.09	179.065
Compound 19	**Cycloastragenol**				
1		**AmA1**	168.9620	4.99	86.640

***** The relatively quantified concentrations are reasonable approximations of the relative amounts of the identified compounds present in each extract (Bhandari and Rajbhandari, 2015; Punyasiri et al., 2015), ****** All the acid compounds are relatively quantified equivalent to the linear curve of gallic acid, ******* All the non-acid compounds are relatively quantified equivalent to the linear curve of quercetin.

Chapter 4
DISCUSSION

1.1 Biochemical phytoprofiling

Medicinal plants are a source of natural components for both traditional systems of medicine such as Ayurveda, Chinese, Unani, and homeopathy as well as modern medicine. Herbal medicine is gaining more popularity as tea extracts, health and fitness supplements, over-the-counter tablets, as well as potential ingredients for the pharmaceutical industry. Most of the herbs are not fit for consumption in their natural state and preparations suitable for administration are made or advised by THPs. Most of these preparations are hot water tea decoctions. In certain cases, use of alcohols such as brandy is also a practice to prepare extracts. As described by the THPs, the herbs used in this study are incubated overnight with alcoholic beverages such as brandy before consumption (Thring and Weitz, 2006). Hence aqueous and ethanolic extractions have been selected for this study. Methanol and ethyl acetate are effective solvents in extracting a wide range of hydrophilic phytocompounds (Dhawan and Gupta, 2017), especially flavonoids and phenols (Siddhuraju and Becker, 2003). Aqueous extractions produce the more hydrophilic constituents for most herbs. However higher pharmacological activity is generally observed in methanolic, ethanolic and ethyl acetate extractions (Dhanani et al., 2017). In this study, except for *Withania somnifera* and *Glycyrrhiza glabra*, all other herbs gave the highest yield with the aqueous extraction, *Inula helenium* having the highest % yield of 55%, followed by *Ocimum basilicum* with 33.57%. The lowest % yield was observed in the methanolic extract of *I.helenium* (0.44%).

Qualitative analysis of the herbs is an essential step as a precursor to *in vitro* studies and analytical fingerprinting of the phytoconstituents. Standard methodologies are followed to analyse the various classes of compounds present in each extract based on precipitate formation and color intensity on addition of specific chemical reagents. As per the results, the extracts of *G.glabra*, *Astragalus membranaceus*, *I.helenium* and *O.basilicum* had prominent phytocompounds such as phenols, alkaloids, tannins, terpenoids and glycosides.

Saponins were observed in *O.basilicum*. High intensity of flavonoids, phenols, alkaloids, terpenoids and glycosides were observed in *G.glabra*, *I.helenium* and *O.basilicum*, some of these compounds could also attribute to their strong aromatic nature (Petkova et al., 2015; Loughrin and Kasperbauer, 2003). Phytochemical composition, antimicrobial and medicinal

properties of *G.glabra*, and compounds isolated from its roots such as glycyrrhizin have previously been reported (Karkanis et al., 2018; Thakur et al., 2016). Saponins, alkaloids, flavonoids as well as phenolic compounds such as liquiritigenin, liquiritin apioside, neoliquiritin apioside, isoliquiritin etc. have been identified (Ammar et al., 2012). Plants from the genus *Inula* being popular medicines in Africa, have been analysed for their bioactive components. Mono- and cyclic sesqui-terpene lactones are some of the major phytocompounds identified in *I.helenium* (Zhao et al., 2006; Seca et al., 2014) along with flavonoids (Yanitskaya et al., 2012). *O.basilicum* extracts have been analysed to determine triterpenoid saponins (Habib et al., 2016), alkaloids, flavonoids, saponins and anthraquinones from the methanolic extract as well as glycosides, tannins, terpenoids, phenols and phlobatatannins in the aqueous extract (Fakhroo and Sreerama, 2016). Major saponins such as astragaloside and astramembranoside were identified from the ethanolic extract of *Astragalus* roots (Ju et al., 2008). Other phytochemical studies investigated the flavonoid derivatives and lignans present in this herb (Bratkov et al., 2016; Lee et al., 2008). The biochemical profile of the some of the herbs in this study showed the presence of more compounds such as phytosteroids in *W.somnifera* and *O.basilicum*. The ethyl acetate extract of *O.basilicum* comprised of all the phytocompounds including steroids, in trace amounts. The presence of more coumarins and alkaloids in the extracts of *A.membranaceus* and *I.helenium* compared to the other class of phytocompounds was new data. Previous studies have not reported comparative biochemical analysis of all extracts for each herb, including the ethyl acetate extract.

As illustrated in the phyto-CYP interaction assessment prediction model (fig. 3), flavonoids have been previously reported to have inducing as well as inhibitory effects on CYPs. Common phytoconstituents such as rutin, isoquercitrin and quercetin have inducing effects on many CYPs, while dimethoxy flavanones inhibit certain CYPs. Since the intensity of flavonoids was high in *G.glabra*, *I.helenium* and *O.basilicum*, the prediction model would associate these herbs to have inhibitory or inducing effects on the CYPs. Saponins as well as glycosides are known to have moderate inhibitory effects on certain CYPs (3A4, 1A2), which could indicate that *A.membranaceus* and *O.basilicum* also have more inhibitory effect based on the type of extract used, attributing to the solvent extraction. Extracts of *Althaea officinalis* had mostly flavonoids and glycosides; some of these compounds could make them potential candidates to cause HDI. From the qualitative analysis, the herbs that would cause strong interactive effect with the CYPs based on the presence of the various classes of phytocompounds where ranked as follows: 1. *O.basilicum*, 2. *G.glabra*, 3. *A.membranaceus*, 4. *I.helenium*, 5. *W.somnifera* and *A.officinalis*.

From the biochemical analysis, the intensity of phytocompounds present in *W.somnifera* and *A.officinalis* was comparatively less, indicating their potential to cause moderate yet clinically relevant HDI.

1.2 CYP inhibition

HLM assays for *in vitro* inhibitory evaluation of herbs on CYP metabolism is one of the most convenient methodologies, recommended by FDA (FDA Clinical Pharmacology Guidelines, 2012). Mostly the assay samples are analysed using HPLC and LC-MS techniques, for quantification of the metabolites, the parent drug and the internal standard. Two methods using different solvents were developed to separate and quantify EFV, E8H and NEO as well as RIF, 25ODESRIF and NEO. The assay yielded well-resolved peaks for all the analytes with good accuracy, precision and recovery, within a short run time. As per literature, biphenyl columns are preferable for the analysis of cyclic compounds (Powell and D'Arcy, 2013); hence a comparative study was done to analyse EFV and its metabolite using a biphenyl column as well as a C-18 column. Good separation of analytes was obtained on both columns, however shorter run time was observed in the C-18 column. For RIF, a column with comparatively larger diameter was preferred and the C-18 Luna column gave good separation.

The HLM assay samples were analysed using the same methods as the standards, and a gradient elution gave better results, and negated the interference of other metabolites or co-factors from the assay. For the HLM and time variant assays, consistent results were observed for both EFV and RIF. Based on the rate of metabolite formed, as well as the time of incubation, 0.5 mg/mL and 0.25 mg/mL protein concentrations were selected for EFV and RIF assays, respectively. EFV has a higher binding affinity to CYP2B6 with reported K_m values ranging from 13-20 μM (Ward et al., 2003; Ogburn et al., 2010) compared to other substrates such as bupropion (Xu and Desta, 2013). Other reported K_m values of EFV include 3.2-8.8 μM (in CYP2B6.1/2B6.6 isoenzymes; Xu et al., 2012) and 14.3-15.9 μM (in CYP2B6/2B6.4 isoenzymes; Bumpus et al., 2006). The K_m value expressed for EFV-HLM in this study (15.08 μM) was in agreement with the latter values reported in literature.

Previously reported values of RIF K_m in transporters SLCO1B1 (OATP1B1, OATP-C, OATP2, LST-1) in HeLa-OATP1B1, OATP1B1-expressing oocytes, and SLCO1B3 (OATP1B3, OATP8) in OATP1B3-expressing oocytes are 1.5 μM, 13 μM and 2.3 μM (Tirona et al., 2003; Vavricka et al., 2002) respectively. High rifampicin hydrolase activity was observed in

recombinant human arylacetamide deacetylase (AADAC) and human liver and jejunum microsomes with K_m values 0.2 mM, 0.4 mM and 0.3 mM, respectively (Kobayashi et al., 2012). In a study on *Staphylococcus aureus*, based on the accumulation of ^{14}C-Rifampicin, the K_m value expressed was 0.06 μM (Williams and Piddock, 1998).

The *in vitro* data showed that 25ODESRIF is the primary metabolite, which concurs with previous studies (Seng et al., 2015; Panchagnula et al., 1999). However since RIF is conventionally used as an inducer of CYP3A4 with other drugs, no substantial *in vitro* data is available on the kinetics of RIF (K_m and V_{max}) based on the formation of 25ODESRIF using HLMs.

In the two-point screening assays all herbs were screened for inhibitory activity against CYP2B6 and the metabolism pathway of RIF, since the THP advises using combination of these herbs in scenarios of HIV patients with TB. The results showed that except for ObA1 (affected only RIF metabolism), all extracts of *Ocimum basilicum* and *Inula helenium* inhibited CYP2B6-mediated metabolism of EFV and formation of 25ODESRIF from RIF. Among the other herb extracts including ObA1 there was an increase in the rate of formation of 25ODESRIF at both concentrations (20, 200 μg/mL). For the extracts of *I.helenium*, the activity was concentration-dependent against CYP2B6; IhA1, IhM2 and IhEtA4 inhibited CYP2B6 at higher concentration (200 μg/mL). IhM2 reduced the activity to 30% at 200 μg/mL, whereas IhE3 reduced the activity to 35% at both 20 and 200 μg/mL showing that the ethanolic extract inhibitory effect was not concentration-dependent and was the most potent amongst all the *Inula* extracts. IhE3 has a similar inhibitory effect on rifampicin metabolism also. AmE3 was the only other extract that showed concentration-independent inhibitory effect on CYP2B6. This proved that different solvent extractions had different effects on the activity of the CYPs because the polar/non-polar nature of the solvents whereby different phytoconstituents get extracted in each solvent. ObM2, ObE3, IhM2 and IhE3 strongly inhibited CYP2B6 activity. The ethyl extracts of *A.membranaceus* and *W.somnifera* also inhibited CYP2B6.

The *in vitro* activation of CYP-mediated metabolism of certain substrates has been analysed and reported previously (Tracy, 2006; Hutzler et al., 2003; Atkins et al., 2001; Korzekwa et al., 1998); however the relevance of such CYP modulations *in vivo* is still uncertain. CYP activation is not associated with the increase in mRNA expression and protein levels as in induction, but only an enhancement in the enzyme activity. This enzyme modulation is

measured by the amount of metabolite formed by the CYP from its relevant substrate. In this study the aqueous, methanolic and ethanolic extracts of *Glycyrrhiza glabra* activated CYP2B6 metabolism of efavirenz, increasing the remaining activity by 200% at a higher concentration (200 μg/mL). The aqueous extract of *W.somnifera* increased CYP2B6 metabolism by 195% at a concentration of 20 μg/mL. Similarly, an increase in the metabolism of rifampicin was noticed for many extracts such as ObA1 (170% at 20 μg/mL), AmA1 (143% at 200 μg/mL), GgA1 (136% at 200 μg/mL), AmM2 (146% at 200 μg/mL), AmE3 (122% at 200 μg/mL) and AoE3 AmM2 (118% at 200 μg/mL). A possible reason explaining this mechanism of CYP activation would be the presence of multiple binding sites at the active site of the enzyme (Tracy, 2006; Atkins et al., 2001; Korzekwa et al., 1998). The activation of CYP2B6 and the enzymes involved in rifampicin metabolism could be due to the ability of the phytoconstituents present in these extracts, to bind to the active site of these enzymes thereby inducing conformational changes, thereby increasing the activity. CYP3A4 activation with similar speculations was reported previously, where an increase in the metabolism of dextrorphan and the formation of 3-methoxymorphinan was observed in microsomes treated with *Ferula asafetida* extracts (Al-Jenoobi et al., 2014). Furthermore, such CYP modulations are concentration-dependant.

In the IC_{50} screening, AmEtA4 exhibited strong inhibition of CYP2B6 (29.70 μg/mL) followed by ObM2 (36.07 μg/mL), and IhM2 (42.79 μg/mL), while all other extracts had IC_{50}values > 50 μg/mL. The positive control TICL inhibited CYP2B6 with an IC_{50} value 14.47 μg/mL (54.88 μM). In the case of rifampicin, ObE3 was a potent inhibitor of its metabolism with an IC_{50} value of 8.94 μg/mL, followed by IhE3 (18.58 μg/mL) and ObM2 (31 μg/mL). The ethanolic and methanolic extracts of *I.helenium* showed more inhibition in the metabolism of EFV and RIF compared to its aqueous extract. Comparatively NELF inhibited RIF biotransformation to 25ODESRIF, with an IC_{50} value of 5.44 μg/mL (9.59 μM). NELF was used as an inhibitor control for RIF pathway based on the assumption that certain CYP inhibitors could also inhibit esterases (Polsky-Fisher et al., 2006). The IC_{50} curves for some of the extracts also showed that a proportion of enzyme activity was not inhibited by increased concentrations of the extract. For example, in the 2-point screening against CYP2B6, IhM2 reduced the activity to 30% at 200 μg/mL. However in the IC_{50} curve, the enzyme activity was reduced to less than 50% at a concentration of 100 μg/mL, beyond which the rate of inhibition was less. This indicates that the 2-point screening data is only a relative measure of the inhibitory effect of an extract at two different concentrations (ten-fold difference as in this case) and does not always correlate with the observed IC_{50} data. Similarly, ObE3 and IhE3 showed most activity for a concentration

range from 0-100 μg/mL. At higher concentrations (>100 μg/mL) the IC_{50} curve did not show a drop in the % of remaining activity. In contrast the IC_{50} curve for IhA1 showed weaker inhibition till the concentration range from 0-125 μg/mL (IC_{50}122 μg/mL); between 150 and 200 μg/mL concentrations of this extract, the inhibition curve dipped further reducing the remaining activity to 20%.

Some of the previously reported IC_{50} values of ticlopidine are 0.32 μM (buproprion CYP2B6; Turpeinen, 2004), 2.1 μM (2C19, Chang et al., 2009); 163 μM (TDI for NADPH in HLM; Cerep app. notes, 2010), 12.4-50 μM (for various CYPS; Hagihara et al., 2008). Comparatively, the IC_{50} value obtained in this study for ticlopidine (54.88 μM) was within the range reported earlier of 12.4-55 μM (Choi et al., 2011; Hagihara et al., 2008). In the literature, IC_{50} values for nelfinavir have been reported as 1.90 μM (on CYP3A4 isoform; Granfors et al., 2006), 11-60 μM (on UGTs), 8.4 μM, 2.7 μM (UGT1A1, HLM) (Zhang et al., 2005) and 2.7 μM, 5.1 μM (in cell culture assays; Mahdi et al., 2015).The IC_{50} value of nelfinavir in this study (9.59 μM) was slightly higher than previously reported for an HLM assay (2.7 μM); however the assay conditions and substrates were different; in the latter bilirubin was the substrate and nelfinavir was used as an inhibitor of bilirubin glucuronidation. As per the FDA guidelines (Clinical Pharmacology, 2012) there are no selective *in vitro* inhibitors available for CYP2B6-mediated metabolism; phencyclidine, thiotepa and ticlopidine have been used as time-dependent inhibitors.

TDI is an MBI process where the potency of the inhibitor increases upon prolonged exposure to the enzyme during the pre-incubation period, and the potential formation of metabolites that possess stronger inhibitory activity than the parent molecule to inactivate the enzyme through irreversible binding. The inactivated enzyme has to be replaced by newly synthesized protein to restore activity (*de novo* synthesis) (Meibohm and Zhou, 2013). Since most of the herbal medications are consumed on a daily basis, the inhibition observed could increase over time. Hence all extracts showing inhibition were screened for TDI IC_{50} shift-fold, on preincubation with NADPH, and it was observed that some of the extracts that were not potent inhibitors of CYP2B6 and CYP3A4, had a TDI effect on their activity thereby lowering the IC_{50} value. TDI results in a prolonged interaction with the metabolism of the involved drugs, such as efavirenz in this case. Similar studies have shown that other drugs such as isoniazid have strong TDI effect on the metabolism of efavirenz by CYP2B6 (Court at.al, 2014).

Almost all extracts of *I.helenium* had a lower TDI IC_{50} value on both CYP2B6 and esterase-mediated metabolism of RIF, except for IhA1, IhE3 and IhEtA4 (only showed TDI on 25ODESRIF formation). IhM2 showed most TDI effect on both the enzymes (33 μg/mL and 23.11 μg/mL respectively). ObA1 and ObM2 had strong TDI effect on CYP2B6. ObM2 had the highest TDI shift-fold (> 7). Comparatively both positive controls showed TDI too, the shift-fold observed was >1.5. A possible reason for the TDI effect of most of the herbal extracts could be attributed to the rate of formation of reactive secondary metabolites on preincubation. Extracts such as WsM2, AmE3, ObM2, IhE3 (CYP2B6) and IhM2, IhE3 (RIF-esterases) showed more TDI at lower concentrations on pre-incubation with NADPH, which could be due to the formation of more reactive metabolites at higher concentration, that could have resulted in the uncoupling of the 'active' inhibitory phytoconstituent from the binding site of the enzyme, thereby reducing its TDI (Fowler and Zhang, 2008). At lower concentrations, there might be less secondary metabolites to interfere with the binding of the active constituent to the enzyme thereby showing more TDI.

IhA1, IhM2 (CYP2B6) and IhM2 (RIF-esterases) showed more TDI at higher concentrations on preincubation with NADPH which could indicate the possibility of MBI. TICL and NELF also inhibited more at higher concentrations indicating their MBI of both the CYPs.

1.2.1 Phytochemical analysis of extracts causing inhibition

The use of pure compounds of particular phytochemical groups, as standard reference markers based on their important properties, is the "gold standard" for analytical fingerprinting (Zöllner and Schwarz 2013). The LC-MS/ PDA analysis used quercetin and gallic acid as reference markers. In other instances where pure reference standards for the various phytocompounds were not available, tentative identifications were made for the compounds present in each extract on the basis of their accurate mass, elemental composition and the corresponding MS fragmentation spectra from the literature, and the UV maxima.

The LC-MS/PDA analysis approach outlined the following principles for identification/relative quantification of a phytocompound: *1*. Accurate masses of the major peaks from the chromatograms, and their product ion transitions (MS/MS fragments) *2*. Relative retention times and UV maxima *3*. Comparison of the masses and their transitions to known compound matches in spectral database repositories and literature, and *4.* Based on specific methods and conditions, each reference standard gave an optimum peak resolution at a specific retention time

for the positive and negative scan modes, and this was used to set up a calibration curve for relative quantification of the identified phytoconstituent in the herbal extract.

For all the extracts that showed inhibitory activity on CYP2B6 and RIF metabolism in HLMs, the LC-MS analysis both in positive and negative scan modes mainly helped in identifying the polyphenols and flavonoids in these extracts, and relatively quantifying the major compounds using the calibration curves set up for the reference standards gallic acid and quercetin. For *O.basilicum* the major polyphenol identified was rosmarinic acid, which is a potential inducer of certain CYPs. This aromatic compound is a major constituent in the fragrant essential oil from *basil*. The other major compounds included salvigenin, tartaric, isocitric, caftaric and chicoric acids and the flavonoid rutin, apigenin-7-O-glucoside along with eupatorin. These compounds had shown varying levels of inhibition on the CYPs and could have contributed to the activity shown by *basil* extracts in HLM on CYP2B6 and RIF metabolism. In previous studies, salvigenin was reported to have a moderate inhibiting effect on the activity of CYP3A enzymes (Quintieri et al., 2007). The caftaric and chicoric acid present in *Echinacea*, is known to interfere with CYP activity, especially inhibiting CYP3A4 (Cichello et al., 2016; Bossaer and Odle, 2012). Rutin had previously been reported to inhibit CYP3A4 in human hepatoma cells (Karakurt, 2016). Apigenin derivatives have also shown an inhibitory effect on CYP3A4 activity (Tang et al., 2017, Basheer and Kerem, 2015). Rosmarinic acid induced the *in vitro* activity of CYP1A, 2B, and 3A in rat HepG2/C3A and MH1C1 cells (Cho and Yoon, 2015) and jasmonic acid strongly induced CYP mRNA in biotransformation of (--)-isopiperitenone (Son et al., 1998). Highest concentrations (mg/L equivalents of quercetin/ gallic acid) of salvigenin, eupatorin, and caffeic acids were observed in the ethanolic extract; hence it caused strong inhibition of both the RIF metabolism pathway, compared to the aqueous and methanolic extracts. Eupatorin has previously been reported to inhibit CYP1A2 with IC_{50} value of 50.8 μM (Pan et al. 2014), and strongly inhibit the *in vitro* proliferation of MDA-MB-468 human breast cancer cells that express CYP1A1 (Androutsopoulos et al., 2008). Furan-2(3H)-one compound, a moderate inhibitor of CYP1A2 (Martikainen, 2012), was observed in the aqueous extract. The methanolic extract had much more phytocompounds including rutin and medioresinol, which could explain its inhibitory effect on both CYP2B6 and RIF metabolism. This matched the HDI data (section 1.7.6) on the inhibitory characteristics of *basil* with other CYPs.

The fingerprint results indicated the presence of compounds that have mostly inhibitory effect on CYP activity. Compounds such as salvigenin, tartaric, isocitric, caftaric and chicoric acids, apigenin-7-O-glucoside, eupatorin, furan-2(3H)-one and rutin are capable of inhibiting CYP-

mediated metabolism (Ashour et al., 2017; Pan et al. 2014; Martikainen, 2012; Mandery et al., 2010; Androutsopoulos et al., 2008; Quintieri et al., 2007; Budzinski et al., 2000), which could explain the inhibitory effects of *basil* extracts on CYP2B6 and RIF metabolism.

For the extracts of *I.helenium*, the phytochemical profile comprised more of germacrane sesquiterpenoids and lactones, along with quinic acid. Tanacetol A was the major compound detected, along with macrophyllilactone B. Germacrane sesquiterpenoids of the similar category have shown inhibitory potential on CYP2B6 and CYP3A4 activities *in vitro*, with IC_{50} values below 10 μM (Pimkaew et al., 2013), which could explain as to how *Inula* extracts are the strong inhibitors of both CYP2B6 and RIF metabolism. The positive scan showed the presence of isoalantolactone (helenin) which has good inhibitory potential on CYPs such as CYP2C19 (IC_{50} 38µM, Kong et al., 2014). Epigallocatechin gallate was mostly present in IhM2 along with the sesquiterpene alcohol - tanacetol A (Špičáková et al., 2017), which could explain as to why this extract inhibits both metabolism pathways. Epigallocatechin gallate has previously been isolated from green tea extracts and identified as an inhibitor of CYP2B6, CYP2C8, CYP2C19, CYP2D6 and CYP3A (Misaka et al., 2013) and CYP2A6, CYP2C19 and CYP2E1 (Muto et al., 2001). Other compounds identified in the aqueous extract included chlorogenic acid, cryptochlorogenic acid and 5-caffeoylquinic acid. Chlorogenic acid compounds have previously been reported to inhibit CYP2C8, CYP2C9, CYP2B6, CYP2C19, CYP1A2 and CYP3A4 in HLMs (Kang et al., 2016). The synergistic effect of all these compounds or the influence of one single compound could be responsible for the inhibitory effect of *Inula* extracts. Alantolactones and isoalantolactones from *Inula* showed strong inhibitory effects on CYP3A4 and CYP2C19 activity (Qin et al., 2015, Kong et al., 2015). Apigenin detected in the ethanolic extract, has previously been reported to have a strong CYP inhibitory effect, comparable to that of known inhibitors such as ketoconazole (Steuck et al., 2016).

Based on these results, there is substantial phytochemical evidence of the presence of compounds in the *Inula* extracts which may have an inhibitory effect on CYP2B6 or on RIF metabolism. Phytocompounds such as tanacetol A, chlorogenic acid, cryptochlorogenic acid, 5-caffeoylquinic acid, epigallocatechin gallate, helenin (isoalantolactone) and apigenin could be involved in the inhibition shown in HLMs (section 3.3.4). Furthermore, pure alantolactones isolated from *Inula* have been shown to inhibit the activity of CYP3A4 and CYP2C19, as discussed in the literature review.

W.somnifera was ranked last in the list of herbs as per their biochemical profiles (section 1.1) to have potential in causing HDI as per the phyto-CYP assessment model; however the methanol and ethyl acetate extracts inhibited CYP2B6 metabolism in HLMs, when compared to the other herbs that were ranked higher for having more potential of causing inhibition. The ethyl acetate extract contained higher concentrations of withaperuvin, withanolides, eucommiol and vicosalactone B. Isopelletierine was found only in the methanol extract, along with trace amounts of withaferin derivative. Herbal constituents from these chemical classes including isopelletierine have previously been reported to affect CYP1A2 and CYP3A4 (Hidaka et al., 2005; Yu et al., 2014) activity. These compounds could have been responsible for the significant inhibitory activity shown by *Withania* extracts on CYP2B6 in this study. In previous studies, the principal phytoconstituents from *Withania* (Withaferin-A, Withanolide-A and Withanoside-IV) and crude methanolic extracts did not having any significant inhibitory effect on the activity of CYPs such as CYP3A4, CYP1A and CYP2D6 in both rat and human liver microsomes (Savai et al., 2015; Savai et al., 2014; Savai et al., 2013). In this scenario, a possible explanation would be that the previous studies did not explore the effect of *Withania* on CYP2B6, even though this herb is used by THPs for HIV/AIDS and many other diseases. Compounds such as withaferin A, salvigenin and isopelletierine detected in the phytochemical screening extracts could be involved in the inhibitory effect on CYP2B6, alone or in combination with some or all withanolides or withanosides.

The previous studies on the activity of *W.somnifera* extracts used the roots available in India (Savai et al., 2015; Savai et al., 2014; Savai et al., 2013). In all the studies, no significant inhibition on the CYPs was observed for *Withania*. In this study, the extracts from the roots of *W.somnifera* obtained from South Africa showed significant inhibition of CYP2B6. This could be attributed to the fact that the phytochemical composition of any herb depends on the type of plant, its family characteristics, the geographical conditions and pedodiversity of its place of origin (Gambetta et al., 2017; Machado et al., 2016; Pirbalouti and Mohammadi, 2013). For example, evidence-based studies previously reported the compound withaferin A in *Withania somnifera* (L.) Dunal, being present in most of the Indian chemotypes, but absent in other non-Indian varieties except two chemotypes, one from South Africa and the other from Israel (Kaul et al., 2009).

A.membranaceus inhibited CYP2B6 metabolism, and like *Withania* the ethyl acetate extract was more potent than the ethanolic extract, whereas the methanolic extract showed no inhibition

at all. Calycosin (7,3'-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone) were prominent in all extracts. Herbal formulations containing these isoflavonoids significantly increased the mRNA expression of hepatic CYP1A2 and CYP2B1/2 in female rats (Jin et al., 2017). Furthermore, formononetin moderately inhibited CYPs such as CYP3A4 and CYP2C9 (Kopečná-Zapletalová et al., 2016). Astragaloside I, II, IV and ononin were detected in the methanolic and ethanolic extracts (using the positive mode scan). Astragaloside IV inhibited the activity of CYP3A4 and CYP2C9 in rat liver microsomes (Wei et al., 2014; Shan et al., 2012). Ononin was one of the compounds isolated from the extracts of plants used in TCM that inhibited CYP3A4 activity (Ashour et al., 2017). Benzopyran derivatives (3',4',7-trimethoxyflavan) and salvigenin were observed in the ethyl acetate extract, which could have contributed to its inhibitory effect on CYPs.

The inhibitory effect of *Astragalus* extracts on CYP2B6 could be due to presence of the various astragalosides as well as other flavonoids such as calycosin, formononetin and ononin. Literature review on *Astragalus* describes the inhibitory effect of the pure astragalosides isolated from the roots of this herb (astragaloside IV, cycloastragenol) and not all its crude extract forms (aqueous, methanolic, ethanolic and ethyl acetate).

A.membranaceus, G.glabra and *W.somnifera* are few herbs from which many pure molecules have been isolated and identified. The predictions of possible compounds and a literature review of their previous reported interactions with CYPs formed the basis for this analysis, to link the fingerprint of each extract to the inhibition/ induction assays as a potential inhibitor or inducer of CYP2B6 and RIF metabolism pathway. As per literature review, some of the pure molecules from these herbs such as astragaloside, cycloastragenol and glycyrrhizin are inhibitors of the CYPs (Qin et al., 2015; Kong et al., 2014; Wei et al., 2014; Shan et al., 2012; Zhao et al., 2012; Kent et al., 2002). This study has provided substantial data on the potential of the crude extracts (comprising of several active compounds) of these herbs to interact with the CYPs.

1.3 CYP induction

Understanding the induction of CYPs is critical especially in scenarios where herbal medicines are used, as a basis for proper clinical studies on their effects on CYP-mediated metabolism. Compared to the other CYPs, CYP3A4 is more efficiently induced (Dogra et al., 1998; Denison and Whitlock, 1995; Waxman and Azaroff, 1992) and was chosen for the mRNA expression study along with CYP2B6. Using this methodology, the major emphasis was on determining the cytotoxicity concentrations (CTC_{50}) of each herbal extract by screening various concentrations

(1000.00-31.25 µg/mL) and analysing the CYP2B6/CYP3A4 mRNA expression in HepG2 cell lines using this concentration. Based on this data, the fold responses for both the CYPs were calculated and compared to the (negative) cell control and a positive control.

For almost all extracts, the aqueous and ethyl acetate extracts had higher CTC_{50} values than the ethanolic and methanolic extracts indicating a higher potency of the phytoconstituents present in these solvent extracts.

For CYP2B6, the methanolic extract of *O.basilicum* and the aqueous extract of *A.membranaceus* caused moderate induction compared to the cell control (no inducer). The ethanolic extract of *basil* showed moderate induction which could be attributed to the presence of rosmarinic acid both extracts, at varying levels. The ethanolic extract of *G.glabra* showed moderate induction of CYP2B6 which could be due to the presence of compounds such as glycyrrhizin, or the synergistic effect of the licochalcones and licosaponins. However, none of the extracts showed 0.5-fold shift (50% increase) in the levels of gene expression when compared to the positive control, dexamethasone. The total amount of hepatic CYP3A is 33% compared to only 0.2% for total CYP2B6 (Michaels and Wang, 2014). Previous evaluations on CYP2B6 inductions and DDI predictions have shown that the magnitude of CYP3A4 induction both *in vitro* and *in vivo* was found to be higher than CYP2B6 (Fahmi et al., 2016).

All extracts screened for CYP3A4 induction, except for a few ethyl acetate extracts, showed moderate induction of mRNA expression in HepG2 cells. The methanolic and ethanolic extracts of *A.officinalis* showed most activity with 48%-fold-response shift. This was different from the phyto-CYP prediction model ranking based on the biochemical tests, where *Althaea* extracts showed lesser intensity of phytocompounds. The *Althaea* extracts showed no inhibitory effect on CYP2B6 and RIF metabolism, indicating that the principle constituent has more of an inducing effect on the CYPs. The methanolic extract of *basil* and the ethanolic extract of *G.glabra* induced CYP2B6 moderately with an increase in fold-response by 38 and 33%, respectively.

The aqueous extract of *Astragalus* (at CTC_{50} concentration 315 µg/mL) induced both the CYPs equally with a 36%-fold response increase compared to the cell control (no inducer). This was slightly different from all other aqueous extracts screened, where the relative induction levels were comparatively less. This could be attributed to the fact that the principle constituent in

Astragalus causing induction is extracted most in the aqueous solvent and has a stronger inducing effect on both CYP2B6 and CYP3A4.

An *in vivo* pharmacokinetic study of co-administered methanolic extract of *Withania somnifera* and phenacetin in male wistar rats, revealed that the crude extract induced CYP1A and lead to an approximate 1.5-fold (31%) decrease in AUC 0-24h ($p < 0.05$) (Savai et al., 2014). In this study, the methanolic extracts of *Withania* showed moderate induction of both CYP2B6 (16%-fold) and CYP3A4 (31%-fold).

In this study, all the extracts of *A.membranaceus* moderately induced CYP3A4. Previous studies reported on the inhibitory effect of its pure molecules (astragalosides and cycloastragenol) on CYP3A4-mediated metabolism, and not on its inducing potential. Cycloastragenol and astragaloside IV were analysed and reported previously as potential inhibitors of CYP3A4, and CYP2C9 (Wei et al., 2014; Shan et al., 2012). In this study, the crude extract of *A.membranaceus* was screened against both CYPs using HepG2 cells for mRNA expression; hence it could be that the synergistic effects of various molecules, or some other unidentified phytoconstituent might be making it a potential inducer of CYP3A4. Another possibility is the presence of glycosides in the extracts as shown in the LC-MS analysis in section 3.6.1, and the synergistic effects of these glycosides could be the reason for the relevant induction observed.

Similarly, *O.basilicum* has been reported as an inhibitor of CYP3A4 (Nguyen et al., 2014). In this study the extracts of *O.basilicum* inhibited CYP3A4, and the ethanolic extract moderately induced CYP3A4 mRNA in HepG2 cells.

In most of the induction studies reported on CYPs, using *in vitro* model (hepatocytes) or animal model (rats), the herbal extract is administered for 2-8 days or prolonged time frame up to 4 weeks (Cho and Yoon, 2015; Komoroski et al., 2004) before analysing the CYP protein expression. In this study, the HepG2 cells were incubated with the herbal extracts for a shorter time period of 24 h, for optimum CYP-mRNA expression (Nagarajappa et al., 2016; Brimer, 2011; Jia and Liu, 2009).

1.3.1 Phytochemical analysis of extracts causing inhibition

A.officinalis extracts were found to have moderate induction potential on CYP3A4. This could be attributed to the high mucilage and fatty acid content within *althaea* roots, especially acids such as trihydroxy-octadecenoic acid. Increased levels of fatty acids are known to induce

hepatic CYP3A4 activity, as reported in an *in vitro* study in HepG2 cell and Fa2N-4 cell lines (Hu et al., 2014). The other prominent compounds included altheahexacosanyl lactone derivatives (n-hexacos-2-enyl-1,5-olide) and phloretin, which could have probably caused the observed induction of CYP3A4 mRNA in HepG2 cells. Polyphenolic apple juice extract consisting of phloretin showed moderate inducing effect on CYP1A1 (Pohl et al., 2006).
The inducing effect shown by the methanolic and ethanolic extracts of *althaea* could be attributed to the presence of compounds such as altheahexacosanyl lactones and phloretin, along with the natural fatty acids present in the mucilage extracted from this herb (Hu et al., 2014). Few of the other known compounds such as 2*B*-hydroxycalamene (altheacalamene), 5,6-dihydroxycoumarin-5-dodecanoate-6*B*-D-glucopyranoside (altheacoumarin glucoside) and *B*–sitosterol were not detected in the LC-MS scans, however the inducing effect could be the synergistic effect of all these compounds. Isolation, characterization and purification of these molecules and separate analysis on HepG2 cells, is warranted.

O.basilicum extracts had rosmarinic acid, along with 12-hydroxyjasmonic acid and fukugetin, which could have added to their inducing capabilities on CYP3A4 mRNA expression (Cho and Yoon, 2015).

The bioactive saponin cycloastragenol was analysed in rat hepatic microsome enzymes to induce CYP2E1. Rats treated with cycloastragenol for 7 days to induce hepatic microsomal enzyme, significantly activated CYP2E1, inhibited CYP3A4 and caused no major effect on CYP2D6, CYP1A2 and CYP2C9 activity (Wei et al., 2014). This compound was detected in the aqueous extract of *Astragalus* and could have been the inducer of CYP2B6, or it could have also been the synergistic effect of all the astragalosides and cycloastragenol along with other molecules like physcion.
Astragalus injections and *astragalus* granules significantly increased the CYP3A1 mRNA expression on the HLMs in rats; along with inducing CYP3A4 reporter gene luciferase activity in the reporter gene study conducted in human intestinal LS174T cells (Zhang et al., 2013). The results of this study matched the inducing potential previously reported for *astragalus* on the CYPs, though comparatively being moderate inducer of CYP2B6 and CYP3A4 in HepG2 cells.

The ethanolic extract *G.glabra* induced CYP3A4 which could be the combined effect of the licochalcones and glycyrrhizin. As reported previously, CYP3A4 was mostly inhibited by the pure molecules isolated from some of these herbs (Wei et al., 2014; Shan et al., 2012; Zhao et

al., 2012; Kent et al., 2002), except for glycyrrhizin (from *liquorice*) which induced CYP3A4 (Tu et al., 2010).In a study previously reported in mice and rats, multiple oral doses of *liquorice* extract during 4 or 10 days induced the mRNA and protein expression of CYP3A and activity of CYP1A2, CYP2B1 and CYP3A (Cho and Yoon, 2015) indicating the similarity in activity of this extract, causing moderate induction on CYP3A4 mRNA expression in HepG2 cell lines in this study. *Liquorice* extracts including glycyrrhizin have been reported to significantly induce hepatic CYP3A- and, to a lesser extent, CYP2B1- and CYP1A2-dependent microsomal monooxygenase activities, as well as 6beta- (CYP3A), 2alpha, 6alpha- (CYP2A1, CYP2B1) and 7alpha-, 16alpha- (CYP2B9) activities in male and female mice (Paolini et al., 1998). The observed induction could be the effect of glycyrrhizin, because most of the licochalcones extracted from *liquorice* previously, have been associated with inhibitory effects on the CYPs (Li et al., 2017).

The inhibitory/ inducing activity observed for the extracts, could be due to the presence of other unidentified molecules or the synergistic effect of the identified compounds together. It cannot be substantiated if a single molecule alone might cause interactions with CYP2B6 and RIF metabolism, or other unidentified phytoconstituents are involved. For e.g., for basil, the methanolic and ethanolic extracts inhibited CYP2B6 and rifampicin metabolism in HLM, and the same extracts induced CYP2B6 and CYP3A4 mRNA expression in HepG2 cells. The inhibitory effect could be due to the presence of caftaric, chlorogenic and chicoric acids (Cichello et al., 2016; Bossaer and Odle, 2012), or rutin alone (Karakurt, 2016), or a combination of all these molecules along with other unidentified molecules present in the extracts. Similarly, the inducing effect could be attributed to the presence of rosmarinic acid alone in the extracts (Cho and Yoon, 2015), or other compounds such as jasmonic acid (Son et al., 1998), or even a combination of both and other unidentified molecules. Previous studies have reported cases of a plant extract having stronger inhibition potential than its single isolated bioactive molecule, where it was explained as the synergistic effect of other active molecules (Kar et al., 2015; Harwansh et al., 2014).

Chapter 5
CONCLUSIONS, STUDY LIMITATIONS AND RECOMMENDATIONS

1.1 Conclusions

The primary objective of this research study was to investigate the possible inhibitory and inducing effects of *Withania somnifera, Glycyrrhiza glabra, Astragalus membranaceus, Inula helenium, Althaea officinalis* and *Ocimum basilicum* on CYP2B6, esterase-mediated metabolism of rifampicin, and CYP3A4. This was achieved in four phases of research: [1] biochemical phytoprofiling of all the extracts to assess their HDI potential, [2]investigating the inhibitory effect of each extract on CYP2B6 and rifampicin metabolism pathway, using human liver microsomes, and ranking the extracts for potential HDI based on IC_{50}s, as well as TDI shift-foldIC_{50}s, [3] investigating the potential of the herbal extracts to induce CYP2B6 and CYP3A4 mRNA expression in HepG2 cell lines using RT-PCR technique based on the CTC_{50} concentrations determined for each extract, and [4] a complete phytochemical analysis of the active herbal extracts using LC-MS/PDA, to understand the constituents present and their potential to interfere with the CYP activity.

Based on the findings of the study, the following conclusions can be drawn.

1.1.1 Biochemical phytoprofiling:

Bioactive compounds present in the herbs were analysed, for their properties to interfere with CYP activity. These included alkaloids, flavonoids, phenols, coumarins, saponins, glycosides, sterols, tannins and terpenoids. Based on the biochemical profiling each herb was ranked for its potential to cause HDI.

In this study, the qualitative screening ranked *Ocimum basilicum* to be most active, and *Withania somnifera* and *Althaea officinalis* to have lesser potential to cause HDI.

1.1.2 HLM Assays, kinetics of efavirenz/ rifampicin and CYP-herb inhibition data:

Based on the solvent extraction each extract from the same herb showed variable activity on the enzyme activity. Though rifampicin is used as an inducer, in this study it was specifically used as a substrate. Using HLM assays and the optimal parameters (K_m, protein concentration), the herbal extracts were screened for activity. As per the study, extracts prepared from hot tea decoctions as practised by the THPs, had less inhibitory

activity on the CYPs compared to the methanolic, ethanolic and ethyl acetate extracts. All the extracts of *O.basilicum* showed strong inhibitory potential as well as TDI, which may be attributed to the fact that for this herb the dried leaves, inflorescence and seeds were used compared to the root extracts of all the other herbs; hence more phytoconstituents such as flavonoids and other secondary metabolites may be present in the essential oil of these extracts (Idris et al., 2017; Mulata et al., 2015; Ugochukwu et al., 2013; Fathiazad et al., 2012).The aqueous and methanolic extracts of *O.basilicum* showed reversible and TDI of CYP2B6, while the methanolic and ethanolic extracts inhibited the formation of 25-O-desacetyl rifampicin. Previous studies reported the inhibitory effect of its ethanolic extract on CYPs such as CYP3A4. However no data is available on its effect on *in vitro* rifampicin metabolism. The ethyl acetate and ethanolic extracts of *A.membranaceus,* and ethanolic extract of *I.helenium* showed strong inhibition of CYP2B6 and rifampicin metabolism, respectively. *O.basilicum, A.membranaceus* and *I.helenium* have high risk to cause HDI in patients on other medications metabolized by CYP2B6 as well as the biotransformation of rifampicin, which might have toxic effects.

The THPs use alcoholic beverages such as brandy for reconstitution of the herbs. Ethyl acetate extractions are used for specific compounds such as TA-65 or secondary metabolites from *A.membranaceus* (Ghasemian-Yadegari et al., 2017). All extracts of *I.helenium* inhibited the investigated enzymes, which could be due to the presence of inulin, alantolactone and isoalantolactone. Only the ethyl acetate and methanolic extracts of *Withania somnifera* inhibited CYP2B6. Rifampicin being the first-line agent in the treatment of drug-susceptible TB could undergo incomplete metabolism due to the inhibition caused by some of the herbs, which might result in toxic effects. Thus *I.helenium* should be used with caution, if co-administered with rifampicin.

O.basilicum showed strong TDI on CYP2B6. Previous *in vitro* studies has shown that NADPH is not a requirement for deacetylation of rifampicin, and that the addition of NADPH to microsomes increased the formation of 25-O-ascetyl rifampicin, which was then attributed to the possible involvement of NADPH-dependent enzymes such as CYPs (Jamis-Dow et al., 1997; Parkinson et al., 1996). In the HLM assays performed in this study NADPH was used in both co-incubation and pre-incubation assays with herbs. It was noticed that both nelfinavir and *O.basilicum* caused strong TDI of the formation

of 25-O-desacetyl rifampicin when pre-incubated with NADPH. This suggests that there could be other enzymes involved in the biotransformation of rifampicin, since exogenous supply of NADPH was not mandatory in the deacetylation of this drug.

Caution must be exercised in patients concurrently taking such medications, especially rifampicin. This finding is particularly relevant for *basil*, considering the fact that it is commonly used as a culinary spice, herbal tea, as well as an essential oil. At low concentrations *basil* extracts could inhibit CYP2B6 and rifampicin metabolism *in vitro* in HLM; if the same or putative concentration levels are reached in the GIT, it could cause adverse HDI in patients consuming *basil*.

1.1.3 mRNA expression assays using RT-PCR for induction analysis:

All extracts screened in this study, showed moderate induction of CYP3A4, while only few extracts induced CYP2B6. The main conclusion of this part of the study was that CYP3A4 was more inducible compared to CYP2B6, which could be attributed to the lesser amount of total CYP2B6 content in the cells as mentioned earlier. This matched previous HDI studies on CYP3A4 (Cho and Yoon, 2015; Hellum et al., 2007; Kent et al., 2002). All extracts of *A.membranaceus* showed moderate induction of CYP3A4 which was in contrast to previous reports of its inhibition of CYP3A4. In the inhibition assays the ethanolic and methanolic extracts of *O.basilicum* inhibited RIF metabolism and CYP2B6 activity in the HLMs, but in the induction assays the methanolic extract induced CYP2B6 mRNA expression, while the ethanolic extract induced CYP3A4. This could be attributed to the various phytoconstituents in *basil* such as rosmarinic acid, rutin and salvigenin, and the two different cell systems (HLM and HepG2), where incubation time points and activity assessment metrics (drug-metabolite levels and mRNA expression) are also different.

The ethanolic and methanolic extracts of *A.officinalis* showed the highest levels of induction (0.48, 0.44-fold responses), among all the extracts screened on CYP3A4. Previously, no published data was available on the screening of *althaea* against CYP3A4 and CYP2B6, for induction studies.

The aqueous extract of *A.membranaceus* showed moderate and equal induction of CYP3A4 and CYP2B6. Caution must be exercised in patients concurrently taking

conventional medications, when using *A.membranaceus* because of its inducing potential on CYP2B6 and CYP3A4, though at moderate levels. *A.officinalis* root extracts are used in preparing the marshmallow confectionaries, and this could also be a factor in its inducing effect on CYP3A4.All the extracts above showed moderate induction of the CYPs (when compared to the positive control).

1.1.4 Phytochemical fingerprinting:

The LC-MS/PDA methodology detailed the fingerprinting further into the peaks masses and their transition product ions combined with the UV maxima, to identify a list of possible compounds present in each herb. It was observed that extracts prepared using methanol, ethanol and ethyl acetate had higher quantities of phytochemicals compared to the aqueous extracts, though the % yield was higher for the aqueous extracts. The relative quantification of the tentatively identified compounds helped predict the varying levels of activities of the different solvent extracts for each herb on HLMs and HepG2 cells, based on literature review. Prominent and previously unreported phytocompounds were identified in the negative mode LC-MS full scan analyses. These compounds were used to link the activity of the extract to the inhibition/ induction assays and the phyto-CYP prediction model (Fig. 3); the assumption was that herbs such as *Ocimum basilicum, Astragalus membranaceus*, and *Inula helenium* would inhibit CYP2B6, and possibly the esterase-mediated metabolism pathway of rifampicin. Phenolic compounds, terpenes and lactones were the most observed compounds in the herbs showing activity.

1.1.5 Impact:

The THPs involved in this study, were very keen on the outcomes of the research, in understanding the potential of the herbs in causing relevant HDI with ARVs and TB drugs, whereby they could also ensure safer use of such herbs and educate their customers of the potential interactions, when co-administered with conventional drugs. The involvement of THPs in such studies is of critical importance in bridging the gap in contemporary health research, especially in African countries.

1.1.6 Overview:

As per the biochemical analysis, it was predicted that herbs such as *O.basilicum, A.membranaceus* and *I.helenium* had the potential to inhibit CYP2B6 and possibly affect esterase-mediated metabolism of rifampicin. Different solvent extracts for the same herb

had different phytochemical compositions which attributed to the fact that some of them may induce CYPs such as CYP3A4. However the inhibition and induction studies showed that each extract had its own potential to interact with the CYPs. The same herb showed inhibition effect on the CYP2B6 and rifampicin pathway, as well as induction of CYP3A4 based on the type of solvent extract used, and this was attributed to the various phytocompounds identified in each solvent extract of the same herb using LC-MS fingerprinting. These results link the HDI potential of the herb to its fingerprint, to further investigate, isolate and evaluate the potential of various phytoconstituents to cause both induction and inhibition based on the solvent extraction.

Figure 58 provides an overview of the work carried out in this research study. The study has clearly demonstrated the connection and overlap between the various *in vitro* models used. This study design offers a comprehensive model to investigate the HDI potential of herbal medicine, from various aspects.

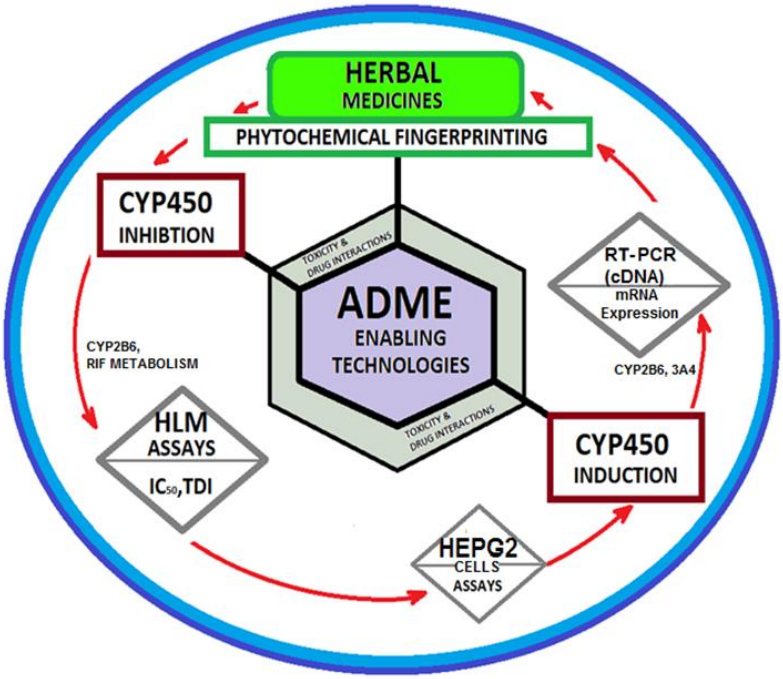


Fig.58 Summary of this research study design and the *in vitro* methods used.

1.2 Limitations of the study

The herbs selected in this study were provided in dried packaged forms by a natural product supplier company. All herbs were from various regions of Africa including South Africa. However there are possibilities that a different batch of these herbals product could be from a different region and hence based on the climatic and environmental conditions, the

phytoconstituents present might vary. It is therefore possible that the same herb from a different region might not show the same CYP interaction as observed in this research study.

All assays were done using the crude extracts. The LC-MS/PDA analysis only provides a potential list of possible phytoconstituents in the extract but not exact molecule responsible for the inhibitory/ inducing effect on the CYPs. Bio-assay guided fractionations are required, and these sub-fractions should be further screened to understand if the interaction is caused by a single fraction or a synergistic effect of two or more fractions. Further analysis of these fractions to isolate the pure molecule is warranted.

For all the HLM assays, the standard deviation (standard error of the mean) for the data points for few extracts show high variance. This could be attributed to external factors, which might have interfered with the assays. All assays including duplicates were done in different test tubes; pipetting the assay termination solvent (acetonitrile) from one tube to another might have had time variance of few seconds or it could have also been due to the loss of metabolite due to degradation, during the prolonged HPLC runs.

All extractions performed in this study follow a pattern of extraction conditions such as temperature, solvent and duration. These factors would ultimately determine the amount of phytoconstituents present in the extract. Roto-evaporation and freeze-drying can have an effect on the extract composition, compared to traditional (non-standardized) methodologies followed by the herbal practitioners. Hence the levels of interactions seen *in vitro* might not be the same *in vivo* in human subjects.

The rifampicin metabolism pathway putforth in this study needs to be analysed and evaluated further, to understand the levels to which B-esterases are involved in this pathway and also investigate any potential involvement of CYPs in this pathway. Currently there is very little information available on this metabolic pathway, in order to fully appreciate the relevance of the findings in this study.

HLMs and HepG2 cells were used as part of *in vitro* model in this study and in all these assays the herbal extracts were in direct contact with the enzymes as well as the substrates, and the cell lines. However in an *in vivo* system, there are many other factors involved such as redistribution through the tissues, concentration differential between tissues, presence of natural barriers such

as varying capillary bed permeability, the epithelial membrane barrier, apical membrane barrier in passive transcellular transport etc. as well as plasma protein binding, sub-epithelial blood flow and intestinal pH (El-Kattan and Varma, 2012; Gavhane and Yadav, 2012). The amount of phytoconstituents that enter the GIT, is dependent on various factors such as intestinal fluid composition, GIT transit time, disease state and dosage form (Fasinu et al., 2014).

The phytochemical analysis using LC-MS, for relative quantification of the tentatively identified compounds uses two standard calibrators, quercetin and gallic acid. The standard curves for both reference compounds are in the range of 6.25-200 mg/L; however there are identified compounds within the plant extracts that have values above the range of the curves (upto values of around 4000 mg/L). These values are relative quantifications based on extrapolations of the standard curves and could have a certain degree of inaccuracy. The acidic compounds were identified relative to gallic acid and the other compounds relative to quercetin. The various classes of compounds detected are likely to ionize in the MS to vastly different degrees, when compared to the standard calibrators. Fold-variations can thus occur in the observed peak area of a compound, when compared to an equivalent amount of gallic acid or quercetin. The matrix effects in the plant extracts can reduce the peak areas significantly and there is no internal standard used in this analysis to measure the impact of such effects on the peak area of a compound. This can also affect the quantification data for the identified compounds. The concentrations represented in this study for the identified compounds are reasonable approximations of the relative amounts, in comparison to the standard calibrators, and are not absolute quantifications (Al Feteisi et al., 2015).

1.3 Recommendations

It is critical to understand HDI especially when CAM is increasingly being included within mainstream integrative healthcare services (Singer and Adams, 2014). In many countries such as USA, India, China, Australia, as well as few of the European countries, CAM has already been incorporated into clinical care, especially in scenarios of therapeutic alliance of CAM with biomedicine, as well as psychological therapies. Traditional systems of medicine being part of a country's heritage, identity and culture always gain more popularity especially among the rural areas. Hence the risk of potential HDI always surface in such cases where herbal medicines are used in combination with conventional drugs. This is where *in vitro* liver-based models for screening such herbal medicines become the best standard for drug interaction studies. However with the increase in consumption of herbal medicines in the form of supplements and tea

extracts, especially among the HIV/TB patients, *in vivo* studies in healthy human subjects are recommended as the next step in understanding HDI of the herbs used as part of this research study. Randomized placebo controlled trial studies would be beneficial to evaluate the safety of consuming such herbal medications, since clinical studies are definitive and considered the "gold standard". Previously such studies have been done for few herbs such as *Lessertia frutescens* in 2015, in South Africa, where the possibility of interaction between isoniazid preventive therapy and *L.frutescens* was identified (Wilson et al., 2015). With trial results available in electronic databases, data extraction and pharmacokinetic/ pharmacodynamics modelling for *in vivo* predictions would be helpful. Alternatively, new *in vitro* strategies using 'Whole Cell' approach where certified human hepatocytes in sandwich-culture provide a fully integrated hepatic cell system with the drug clearance pathways of metabolism and transport, as well as the key regulatory pathways (CAR/PXR) (Jackson et al., 2017). Such systems help in the quantitative assessment of HDI potential of herbal medications. *In vitro* testing on the CYP isoenzymes and transporters as well as clinical trial studies on human subjects will determine if these herbal extracts have additional effects on the CYP-mediated metabolism and the pharmacokinetics of the drugs. The effects of prolonged intake of such herbal medications on the induction of the CYPs should also be investigated. The findings of this study will help to guide planning and designing of clinical trials. The data collected from these trials would help establish the existence of clinically significant HDI in patients, as well as patient counselling by clinicians on the adverse effects of such interactions. Case reports on adverse drug events on these herbs as well as clinical data would also be helpful to connect them to all the relevant *in vitro* findings on these herbs and entries in the FDA Med Watch system, which would ensure post marketing safety assessment of such herbal products (Hussain, 2011).

Furthermore, a critical step forward would be the fractionation, isolation and identification of the active phytoconstituents in these herbal extracts. Bioassay-guided fractionation and structural elucidation of the pure molecules based on NMR spectroscopic analysis would also be beneficial, in drug discovery. These studies would help better in understanding the active 'ingredients' in the herbs as well as their safety or potential toxicity.

The THP involved in this study and I had the opportunity to attend two conferences organized by the Department of Science and Technology and the National Research Foundation of South Africa on indigenous knowledge systems to present my work, along with the views of the THP on how HDI information is very important for evaluating the safety of herbs. The involvement

of THPs in HDI studies is very important; especially to select and identify the herbs, share their knowledge on THMs and methods of treatment, as well as viewpoints on the research outputs.

Chapter 6
REFERENCES

1. Aboyade OM, Styger G, Gibson D, Hughes G (2014). Sutherlandia frutescens: The meeting of science and traditional knowledge. *J Altern Complement Med*, 20(2): 71–76.

2. Ammar NM, El-Hawary SSE, El-anssary AA, Othman N, Galal M, El-Desoky AH (2012). Phytochemical and clinical studies of the bioactive extract of *Glycyrrhiza glabra* L. Family Leguminosae. *Int J Phytomed*, 4(3): 429–436.

3. Androutsopoulos V, Arroo RR, Hall JF, Surichan S, Potter GA (2008). Antiproliferative and cytostatic effects of the natural product eupatorin on MDA-MB-468 human breast cancer cells due to CYP1-mediated metabolism. *Breast Cancer Res*, 10(3): R39.

4. Afemei M, Gille E, Boz I, Toma C, Zamfirache MM (2012). Aspects regarding the qualitative and quantitative phytochemical analysis of the Inula Helenium L. species. *An Stiint Univ Al I Cuza Iasi, Sect IIa Biol veget*, 58(1): 29–34.

5. Akgül A (1989). Volatile oil composition of sweet basil (*Ocimum basilicum* L.) cultivating in Turkey (Short communication) Food / Nahrung, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 33(1): 87–88.

6. Al Feteisi H, Achour B, Barber J, Rostami-Hodjegan A (2015). Choice of LC-MS methods for the absolute quantification of drug-metabolizing enzymes and transporters in human tissue: a comparative cost analysis. *AAPS J*, 17(2): 438–446.

7. Al-Jenoobi FI, Al-Thukair AA, Alam MA, Abbas FA, Al-Mohizea AM, Alkharfy KM, Al-Suwayeh SA (2014). Modulation of CYP2D6 and CYP3A4 metabolic activities by *Ferula asafetida* resin. *Saudi Pharm J*, 22(6): 564–569.

8. Alsanosi SMM, Skiffington C, Padmanabhan S (2014). Chapter 17 - Pharmacokinetic pharmacogenomics, Handbook of pharmacogenomics and stratified medicine, Academic Press Elsevier, San Diego, USA, pp. 341–364.

9. Arbex MA, Varella ML, de Siqueira HR, Fiúza de Mello FA (2010). Antituberculosis drugs: drug interactions, adverse effects, and use in special situations. Part 1: first-line drugs. *J bras Pneumol*, 36: 5.

10. Ashour ML, Youssef FS, Gad HA, Wink M (2017). Inhibition of cytochrome P450 (CYP3A4) activity by extracts from 57 plants used in traditional Chinese medicine (TCM). *Pharmacogn Mag*, 13(50): 300.

11. Atkins WM, Wang RW, Lu AH (2001). Allosteric behavior in cytochrome P450-dependent *in vitro* drug-drug interactions: a prospective based on conformational dynamics. *Chem Res Toxicol*, 14: 338–347.

12. Awortwe C, Bouic PJ, Masimirembwa CM, Rosenkranz B (2014). Inhibition of major drug metabolizing CYPs by common herbal medicines used by HIV/AIDS patients in Africa–implications for herb-drug interactions. *Drug Metab Lett*, 7(2): 83–95.

13. Bailey CJ, Day C (1989). Traditional plant medicines as treatments for diabetes. *Diabetes Care*, 12: 553–64.

14. Bailey CJ, Flatt PR, Marks V (1989). Drugs inducing hypoglycemia. *Pharmacol Ther*, 42: 361–384.

15. Balch P (2006). Prescription for nutritional healing (4th ed.). Avery Penguin Putnam, USA, pp. 236–8.

16. Barry M, Feely J (1990). Enzyme induction and inhibition. *Pharmac Ther*, 48: 71–94.

17. Bartram T (1998). Bartram's Encyclopaedia of Herbal Medicine. London: Robinson Publishing Ltd, UK, ISBN 978-1854875860.

18. Basch E, Ulbricht C, Hammerness P, Vora M (2003). Marshmallow (*Althaea officinalis* L.) monograph. *J Herb Pharmacother*, 3(3): 71–81.

19. Basheer L, Kerem Z (2015). Interactions between CYP3A4 and dietary polyphenols. *Oxid Med Cell Longev*, ID 854015, pp. 15.

20. Bhandari L, Rajbhandari M (2015). Isolation of quercetin from flower petals, estimation of total phenolic, total flavonoid and antioxidant activity of the different parts of *Rhododendron arboreum* Smith. *Scientific World*, 12(12), pp. 34.

21. Bratkov VM, Shkondrov AM, Zdraveva PK, Krasteva IN (2016). Flavonoids from the genus *Astragalus*: phytochemistry and biological activity. *Pharmacogn Rev*, 10(19): 11–32.

22. Brimer L (2011). Chemical Food Safety, CABI Publications, UK, 296p.

23. Bjarnholt N, Li B, D'Alvise J, Janfelt C (2014). Mass spectrometry imaging of plant metabolites – principles and possibilities. *Prod Rep*, 31: 818–837.

24. Bjorkman S (2006). Prediction of cytochrome p450-mediated hepatic drug clearance in neonates, infants and children: how accurate are available scaling methods? *Clin Pharmacokinet*, 45(1): 1–11.

25. Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach RS, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J, Wrighton SA (2003). Pharmaceutical Research and Manufactures of America (PhRMA) Drug Metabolism/Clinical Pharmacology Technical Working Group; FDA Center for Drug Evaluation and Research (CDER). *Drug Metab Dispos*, 31(7): 815–832.

26. Ong CK, Bodeker G, Grundy C, Burford G, Shein K (2005). WHO global atlas of traditional, complementary and alternative medicine, Kobe, Japan: WHO Centre for health development.
Available at:
http://apps.who.int/iris/bitstream/handle/10665/43108/9241562862_map.pdf;jsessionid=C29099B6DF51EDFAF12675CE4068AD73?sequence=1.

27. Bolleddula J, Fitch W, Vareed SK, Nair MG (2012). Identification of metabolites in *Withania somnifera* fruits by liquid chromatography and high-resolution mass spectrometry. *Rapid Commun Mass Spectrom*, 26(11): 1277–90.

28. Bort R, Gomez-Lechon MJ, Castell JV, Jover R (2004). Role of hepatocyte nuclear factor 3 gamma in the expression of human CYP2C genes. *Arch Biochem Biophys*, 426(1): 63–72.

29. Bossaer JB, Odle BL (2012). Probable etoposide interaction with Echinacea. *J Diet Suppl*, 9(2): 90–95.

30. Bozin B, Mimica-Dukic N, Simin N, Anackov G (2006). Characterization of the volatile composition of essential oils of some lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. *J Agric Food Chem*, 54(5): 1822–8.

31. Brandon EF, Raap CD, Meijerman I, Beijnen JH, Schellens JH (2003). An update on *in vitro* test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol Appl Pharmacol*, 189: 233–246.

32. British HIV Association (2011). British HIV Association guidelines for the treatment of TB/HIV coinfection. *HIV Med*, 12: 517–24.

33. Bu HZ, Magis L, Knuth K, Teitelbaum P (2001). High-throughput cytochrome P450 (CYP) inhibition screening via a cassette probe-dosing strategy. VI. Simultaneous evaluation of inhibition potentital of drugs on human hepatic isozymes CYP2A6, 3A4, 2C9, 2D6 and 2E1. *Rapid Commun Mass Spectrom*, 15(10): 741–748.

34. Budzinski JW, Foster BC, Vandenhoek S, Arnason JT (2000). An *in vitro* evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. *Phytomedicine*, 7: 273–82.

35. Bruneton J (1995). Pharmacognosy, phytochemistry, medicinal plants. Edit. Lavoisier, Paris, pp. 503–504.

36. Bumpus NN, Kent UM, Hollenberg PF (2006). Metabolism of efavirenz and 8-hydroxyefavirenz by P450 2B6 leads to inactivation by two distinct mechanisms. *J Pharmacol Exp Ther*, 318(1): 345–51.

37. Cai Y, Baer-Dubowska W, Ashwood-Smith MJ, Ceska O, Tachibana S, DiGiovanni J (1996). Mechanism-based inactivation of hepatic ethoxyresorufin O-dealkylation activity by naturally occurring coumarins. *Chem Res Toxicol*, 9: 729–736.

38. Cantrell CL, Abate L, Fronczek FR, Franzblau SG, Quijano L, Fischer NH (1999). Antimycobacterial eudesmanolides from *Inula helenium* and *Rudbeckia subtomentosa*. *Planta Med*, 65(4): 351–5.

39. Čejchanová J (2011). Inhibition of drug glucuronidation by extracts and constituents of St. John's wort (*Hypericum perforatum*) and Thyme (*Thymus vulgaris*). Charles University in Prague, Fac. of Pharmacy in Hradec Králové, *Würzburg and Hradec Králové*, Thesis ed., pp. 1–64.

40. Cerep app. notes (2010). Cyp-mediated drug-drug interaction, ADME TOX Application notes, Cerep France, Available at:*http://www.cerep.fr/cerep/utilisateurs/pages/Downloads/Documents/Marketing/Pharmacology%20&%20ADME/Application%20notes/2010%20CYP-mediated%20drug-drug%20interaction.pdf*

41. Cermak R, Wolffram S (2006). The potential of flavonoids to influence drug metabolism and pharmacokinetics by local gastrointestinal mechanisms. *Curr Drug Metab*, 7(7): 729–44.

42. Chang JB, Lane ME, Yang M, Heinrich M (2016). A hexa-herbal TCM decoction used to treat skin inflammation: an LC-MS-based phytochemical analysis. *Planta Med*, 82(11-12): 1134–41.

43. Chang SY, Chen C, Yang Z, Rodrigues AD (2009). Further assessment of 17alpha-ethinyl estradiol as an inhibitor of different human cytochrome P450 forms *in vitro*. *Drug Metab Dispos*, 37(8): 1667–1675.

44. Chen G, Li X, Saleri F, Guo M (2016). Analysis of flavonoids in *Rhamnus davurica* and its antiproliferative activities. *Molecules*, 21: 1275.

45. Chen J, Raymond K (2006). Roles of rifampicin in drug-drug interactions: underlying molecular mechanisms involving the nuclear pregnane X receptor. *Ann Clin Microbiol Antimicrob*, 5: 3.

46. Chen P, Lin X, Yang CH, Tang X, Chang YW, Zheng W, Luo L, Xu C, Chen YH (2017). Study on chemical profile and neuroprotective activity of *Myrica rubra* leaf extract. *Molecules*, 22(7): 1226.

47. Cheng J, Fock KM, Chua KL (1988). Reversible hepatic and renal damage from rifampin overdose - a case report. *Singapore Med J*, 29(3): 306–8.

48. Chiang LC, Ng LT, Cheng PW, Chiang W, Lin CC (2005). Antiviral activities of extracts and selected pure constituents of *Ocimum basilicum*. *Clin Exp Pharmacol Physiol*, 32(10): 811–6.

49. Chien CF, Wu YT, Tsai TH (2011). Biological analysis of herbal medicines used for the treatment of liver diseases. *Biomed Chromatogr*, 25(1-2): 21–38.

50. Cichello SA, Yao Q, He XQ (2016). Proliferative activity of a blend of *Echinacea angustifolia* and *Echinacea purpurea* root extracts in human vein epithelial, HeLa, and QBC-939 cell lines, but not in Beas-2b cell lines. *J Tradit Complement Med*, 6(2): 193–197.

51. Cho HJ, Yoon IS (2015). Pharmacokinetic interactions of herbs with cytochrome p450 and P-glycoprotein. *Evid Based Complement Alternat Med*, 2015: 736431.

52. Cho YY, Jeong HU, Kim JH, Lee HS (2014). Effect of honokiol on the induction of drug-metabolizing enzymes in human hepatocytes. *Drug Des Devel*, 8: 2137–2145.

53. Choi JS, Yang JS, Choi DH (2011). Effects of ticlopidine on the pharmacokinetics of diltiazem and its main metabolite, desacetyldiltiazem, in rats. *Biomol ther*, 19(2): 255–260.

54. Collins ML, Irvine B, Tyner D, Fine E, Zayati C, Chang C, Horn T, Ahle D, Detmer J, Shen L-P, Kolberg J, Bushnell S, Urdea MS, Ho DD (1997). A branched DNA signal amplification assay for quantification of nuclei acid targets below 100 molecules/ml. *Nucleic Acids Res*, 25: 2979–2984.

55. Conserve Africa (2002). Medicinal Plants and Natural Products. Conserve Africa Foundation, UK. Available at: *http://www.conserveafrica.org.uk/*.

56. Conte JE Jr, Lin E, Zurlinden E (2000). High-performance liquid chromatographic determination of pyrazinamide in human plasma, bronchoalveolar lavage fluid, and alveolar cells. *J Chromatogr Sci*, 38(1): 33–7.

57. Cooley K, Szczurko O, Perri D, Mills EJ, Bernhardt B, Zhou QI, Seely D (2009). Naturopathic care for anxiety: A randomized controlled trial ISRCTN78958974. *PLoS One*, 4(8): e6628.

58. Cordier W, Steenkamp V (2011). Drug interactions in African herbal remedies. *Drug Metabol Drug Interact*, 26(2): 53–63.

59. Cott JM, Fugh-Berman A (1998). Is St John's Wort (*Hypericum perforatum*) an effective antidepressant? *J Nerv Ment Dis*, 186: 500–01.

60. Court MH, Almutairi FE, Greenblatt DJ, Hazarika S, Sheng H, Klein K, Zanger UM, Bourgea J, Patten CJ, Kwara A (2014). Isoniazid mediates the CYP2B6*6 genotype-dependent interaction between efavirenz and antituberculosis drug therapy through mechanism-based inactivation of CYP2A6. *Antimicrob Agents Chemother*, 58(7): 4145–4152.

61. Cox H, Dickson-Hall L, Jassat W, Moshabela M, Kielmann K, Grant A, Nicol M, Black J, Mlisana K, Vanleeuw L, Loveday M (2017). Drug-resistant tuberculosis in South Africa: history, progress and opportunities for achieving universal access to diagnosis and effective treatment. SAHR – 20 Year Anniversary Edition, Health Systems Trust, SA.

62. Cravotto G, Boffa L, Genzini L, Garella D (2010). Phytotherapeutics: an evaluation of the potential of 1000 plants. *J Clin Pharm Ther*, 35(1): 11–48.

63. Cupp MJ, Tracy TS (1998). Cytochrome P450: new nomenclature and clinical implications. *Am Fam Physician*, 57: 107–116.

64. Daly AK (2006). Significance of the minor cytochrome P450 3A isoforms. *Clin Pharmacokinet*, 45(1): 13–31.

65. de Almeida I, Alviano DS, Vieira DP, Alves PB, Blank AF, Lopes AH, Alviano CS, Rosa Mdo S (2007). Antigiardial activity of *Ocimum basilicum* essential oil. *Parasitol Res*, 101(2): 443–52.

66. de Jesus BB, Schneeberger K, Vera E, Tejera A, Harley CB, Blasco MA (2011). The telomerase activator TA-65 elongates short telomeres and increases health span of adult/old mice without increasing cancer incidence. *Aging Cell*, 10: 604–621.

67. Denison MS, Whitlock JP (1995). Xenobiotic-inducible transcription of cytochrome P450 genes. *J Biol Chem*, 270(31): 18175–8.

68. Denizot F, Lang R (1986). Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability". *J Immunol Methods*, 89(2): 271–7.

69. Desta Z, Flockhart DA (2017). Chapter 18 – Pharmacogenetics of drug metabolism, clinical and translational science (2nd ed.) - Principles of Human Research. Academic Press Elsevier, pp. 327–345.

70. Desta Z, Saussele T, Ward B, Blievernicht J, Li L, Klein K, Flockhart DA, Zanger UM (2007). Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism *in vitro*. *Pharmacogenomics*, 8(6): 547–58.

71. Detersa A, Zippela J, Hellenbranda N, Pappaib D, Possemeyera C, Hensel A (2010). Aqueous extracts and polysaccharides from *marshmallow* roots (*Althea officinalis* L.): cellular internalisation and stimulation of cell physiology of human epithelial cells *in vitro*. *J Ethnopharmacol*, 127: 62–69.

72. Devi A, Devaraj H (2006). Induction and expression of GST-Pi foci in the liver of Cyclophosphamide-administered rats. *Toxicology*, 217(2-3): 120–8.

73. Dhanani T, Shah S, Gajbhiye NA, Kumar S (2017). Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arab J Chem*, 10(1): s1193–s1199.

74. Dharmananda S (2000). The interaction of herbs and drugs, Institute of Traditional Medicine (ITM Online), Available at: *http://www.itmonline.org/arts/herbdrug.htm*.

75. Dhawan D, Gupta J (2017). Comparison of different solvents for phytochemical extraction potential from *Datura metel* plant leaves. *Int J Bio Chem*, 11: 17–22.

76. Dickins M (2004). Induction of cytochrome P450. *Curr Top Med Chem*, 4: 1745–1766.

77. DiPiro JT (1999). Pharmacotherapy: a pathophysiologic approach (4th ed.) Stamford, Conn: Appleton & Lange, USA, pp. 29–30.

78. Directorate Plant Pr. (2013). Medicinal plants of South Africa - Directorate Plant Production, Department: Agriculture, Forestry and Fisheries, SA. Available at: *http://www.daff.gov.za/Daffweb3/Portals/0/Brochures%20and%20Production%20guidelines/Brochure%20Medical%20Plants%20Of%20South%20Africa.pdf*.

79. Dogra SC, Whitelaw ML, May BK (1998).Transcriptional activation of cytochrome P450 genes by different classes of chemical inducers. *Clin Exp Pharmacol Physiol*, 25(1): 1–9.

80. Donato MT, Castell JV (2003). Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism: focus on *in vitro* studies. *Clin Pharmacokinet*, 42(2): 153–78.

81. Du HW, Zhao XL, Zhang AH, Yao L, Zhang YY (2014). Rapid separation, identification and analysis of *Astragalus membranaceus* Fisch using liquid chromatography-tandem mass spectrometry. *J Chromatogr Sci*, 52(3): 226–31.

82. Duke JA (2008). Basil as the Holy Hindu Highness. *Altern Complement Ther*, 14(1): 5–8.

83. Duke JA, Ayensu ES (1985). Medicinal Plants of China, Reference Publications, Michigan, USA.

84. El-Kattan A, Varma M (2012). Oral absorption, intestinal metabolism and human oral bioavailability – Topics on drug metabolism, Dr. James Paxton (Ed.), InTech, Croatia, EU.

85. Engvall E, Perlmann P (1971). Enzyme - linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry*, 8: 871–874.

86. Erickson DA, Mather G, Trager WF, Levy RH, Keirns JJ (1999). Characterization of the *in vitro* biotransformation of the HIV-1 reverse transcriptase inhibitor nevirapine by human hepatic cytochromes P-450. *Drug Metab Dispos*, 27(12): 1488–95.

87. Ezuruike UF, Prieto JM (2014). The use of plants in the traditional management of diabetes in Nigeria: Pharmacological and toxicological considerations, *J Ethnopharmacol*, 155(2): 857–924.

88. Fahmi OA, Shebley M, Palamanda J, Sinz MW, Ramsden D, Einolf HJ, Chen L, Wang H (2016). Evaluation of CYP2B6 induction and prediction of clinical drug-drug interactions: considerations from the IQ consortium induction working group-An industry perspective. *Drug Metab Dispos*, 44(10): 1720–30.

89. Fan L, Wang JC, Jiang F, Tan ZR, Chen Y, Li Q, Zhang W, Wang G, Lei HP, Hu DL, Wang D, Zhou HH (2009). Induction of cytochrome P450 2B6 activity by the herbal medicine baicalin as measured by bupropion hydroxylation. *Eur J Clin Pharmacol*, 65(4): 403–9.

90. Farag MA, Porzel A, Wessjohann LA (2012). Comparative metabolite profiling and fingerprinting of medicinal *licorice* roots using a multiplex approach of GC–MS, LC–MS and 1D NMR techniques. *Phytochemistry*, 76: 60–72.

91. Fasinu PS, Bouic PJ, Rosenkranz B (2014). The inhibitory activity of the extracts of popular medicinal herbs on CYP1A2, 2C9, 2C19 and 3A4 and the implications for herb-drug interaction. *Afr J Tradit Complement Altern Med*, 11(4): 54–61.

92. Fasinu PS, Gutmann H, Schiller H, Bouic PJ, Rosenkranz B (2013). The potential of *Hypoxis hemerocallidea* for herb-drug interaction. *Pharm Biol*, 51(12): 1499–507.

93. Fasinu PS, Gutmann H, Schiller H, James AD, Bouic PJ, Rosenkranz B (2013). The potential of *Sutherlandia frutescens* for herb-drug interaction. *Drug Metab Dispos*, 41(2): 488–97.

94. Fathiazad F, Matlobi A, Khorrami A, Hamedeyazdan S, Soraya H, Hammami M, Maleki-Dizaji N, Garjani A (2012). Phytochemical screening and evaluation of cardioprotective activity of ethanolic extract of *Ocimum basilicum* L. (basil) against isoproterenol induced myocardial infarction in rats. *DARU J Pharm Sci*, 20(1): 87.

95. Fauce SR, Jamieson BD, Chin AC, Mitsuyasu RT, Parish ST, Ng HL, Kitchen CM, Yang OO, Harley CB, Effros RB (2008). Telomerase-based pharmacologic enhancement of antiviral function of human CD8+ T lymphocytes. *J Immunol*, 181(10): 7400–7406.

96. Faucette SR, Wang H, Hamilton GA, Jolley SL, Gilbert D, Lindley C, Yan B, Negishi M, LeCluyse EL (2004). Regulation of CYP2B6 in primary human hepatocytes by prototypical inducers. *Drug Metab Dispos*, 32(3): 348–58.

97. FDA Clinical Pharmacology Guidelines (2012). FDA Guidance for Industry, Drug Interaction Studies – Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), USA.

98. Fiore C, Eisenhut M, Krausse R, Ragazzi E, Pellati D, Armanini D, Bielenberg J (2008). Antiviral effects of *Glycyrrhiza* species. *Phytother Res*, 22(2): 141–148.

99. Flockhart DA (2007). Drug Interactions: Cytochrome P450 drug interaction table. Indiana University School of Medicine, USA.

100. Fowler S, Zhang H (2008). *In vitro* evaluation of reversible and irreversible cytochrome P450 Inhibition: current status on methodologies and their utility for predicting drug–drug interactions. *AAPS J*, 10(2): 410–424.

101. Freimoser FM, Jakob CA, Aebi M, Tuor U (1999). The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Assay is a fast and reliable method for colorimetric determination of fungal cell densities. *Appl Environ Microbiol*, 65: 3727–9.

102. Frye RF (2004). Probing the world of cytochrome P450 enzymes. *Mol Interv*, 4(3): 157–162.

103. Fung EN, Chen YH, Lau YY (2003). Semi-automatic high-throughput determination of plasma protein binding using a 96-well plate filtrate assembly and fast liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, 795(2): 187–194.

104. Galeva N, Yakovlev D, Koen Y, Duzhak T, Alterman M (2003). Direct identification of cytochrome P450 isozymes by matrix - assisted laser desorption/ionization time of flight-based proteomic approach. *Drug Metab Dispos*, 31: 351–355.

105. Gambetta JM, Cozzolino D, Bastian SE, Jeffery DW (2017). Exploring the Effects of geographical origin on the chemical composition and quality grading of *Vitis vinifera* L. cv. chardonnay grapes. *Molecules*, 22, 218.

106. Garcia M, Rager J, Wang Q, Strab R, Hidalgo IJ, Owen A, Li J (2003). Cryopreserved human hepatocytes as alternative *in vitro* model for cytochrome p450 induction studies. *In Vitro Cell Dev Biol Anim*, 39(7): 283–7.

107. Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP, Denison MS (1996). Species - specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8 - tetrachlorodibenzo - p - dioxin - like chemicals. *Fundam Appl Toxicol*, 30: 194–203.

108. Gashaw I, Kirchheiner J, Goldammer M, Bauer S, Seidemann J, Zoller K, Mrozikiewicz PM, Roots I, Brockmöller J (2003). *Clin Pharmacol Ther*, 74(5): 448–57.

109. Gaudineau C, Auclair K (2004). Inhibition of human P450 enzymes by nicotinic acid and nicotinamide. *Biochem Biophys Res Commun*, 317(3): 950–6.

110. Gavhane YN, Yadav AV (2012). Loss of orally administered drugs in GI tract. *Saudi Pharm J*, 20(4): 331–44.

111. Gene: cytochrome P450 (2017). CYP3A4 cytochrome P450 family 3 subfamily A member 4 [Homo sapiens (human)] (updated on 8-Oct-2017). Gene ID: 1576, NCBI, USA.

112. Gene: cytochrome P450 (2017). CYP3A5 cytochrome P450 family 3 subfamily A member 5 [Homo sapiens (human)] (updated on 8-Oct-2017). Gene ID: 1577, NCBI, USA.

113. Ghasemian-Yadegari J, Nazemiyeh H, Hamedeyazdan S, Fathiazad F (2017). Secondary metabolites from the roots of *Astragalus maximus*. *Res J Pharmacog*, 4(2): 31–38.

114. Giraldo R (2002). Brief report - Southern African Development Community (SADC) Meeting on nutrition and HIV/AIDS: 2002, Johannesburg, SA. Available at: *http://www.laleva.cc/choice/aids/aids_giraldo.html*.

115. Glaeser H, Drescher S, Eichelbaum M, Fromm MF (2005). Influence of rifampicin on the expression and function of human intestinal cytochrome P450 enzymes. *Br J Clin Pharmacol*, 59(2): 199–206.

116. Gomez-Lechon MJ, Donato MT, Castell JV, Jover R (2004). Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab*, 5(5): 443–462.

117. Gonzalez FJ and Korzekwa KR (1995). Cytochromes P450 expression systems. *Annu Rev Pharmacol Toxicol*, 35: 369–390.

118. Granfors MT, Wang JS, Kajosaari LI, Laitila J, Neuvonen PJ, Backman JT (2006). Differential inhibition of cytochrome P450 3A4, 3A5 and 3A7 by five human immunodeficiency virus (HIV) protease inhibitors *in vitro*. *Basic Clin Pharmacol Toxicol*, 98(1): 79–85.

119. Guengerich FP (1989). Characterization of human microsomal cytochrome P-450 enzymes. *Annu Rev Pharmacol Toxicol*, 29(1): 241–264.

120. Guengerich FP (2008). Cytochrome p450 and chemical toxicology. *Chem Res Toxicol*, 21(1): 70–83.

121. Guo LQ, Yamazoe Y (2004). Inhibition of cytochrome P450 by furanocoumarins in grapefruit juice and herbal medicines. *Acta Pharmacol Sin*, 25(2): 129-36.

122. Gupta VK, Fatima A, Faridi U, Negi AS, Shanker K, Kumar JK, Rahuja N, Luqman S, Sisodia BS, Saikia D, Darokar MP, Khanuja SP (2008). Antimicrobial potential of *Glycyrrhiza glabra* roots. *J Ethnopharmacol*, 116(2): 377–380.

123. Gurley BJ, Hubbard MA, Williams DK, Thaden J, Tong Y, Gentry WB, Breen P, Carrier DJ, Cheboyina S (2006). Assessing the clinical significance of botanical supplementation on human cytochrome P450 3A activity: comparison of a milk thistle and black cohosh product to rifampin and clarithromycin. *J Clin Pharmacol*, 46: 201–213.

124. Habib S, Bina Shaheen S, Sabira B, Huma Aslam B (2016). Triterpenoid saponins from the methanol extract of *Ocimum basilicum* aerial parts. *J Chem Soc Pakistan*, 38(5): 1014–1017.

125. Hage-Sleiman R, Mroueh M, Daher CF (2011). Pharmacological evaluation of aqueous extract of *Althaea officinalis* flower grown in Lebanon. *Pharm Biol*, 49(3): 327–33.

126. Hagihara K, Nishiya Y, Kurihara A, Kazui M, Farid NA, Ikeda T (2008). Comparison of human cytochrome P450 inhibition by the thienopyridines prasugrel, clopidogrel, and ticlopidine. *Drug Metab Pharmacokinet*, 23(6): 412–420.

127. Hakura A, Suzuki S, Satoh T (1999). Advantage of the use of human liver S9 in the Ames test. *Mutat Res*, 438: 29–36.

128. Harwansh RK, Mukherjee K, Bhadra S, Kar A, Bahadur S, Mitra A, Mukherjee PK (2014). Cytochrome P450 inhibitory potential and RP-HPLC standardization of *trikatu* - a *Rasayana* from Indian Ayurveda. *J Ethnopharmacol*, 153(3): 674–81.

129. Hasani-Ranjbar S, Nayebi N, Moradi L, Mehri A, Larijani B, Abdollahi M (2010). The efficacy and safety of herbal medicines used in the treatment of hyperlipidemia; a systematic review. *Curr Pharm Des*, 16(26): 2935–47.

130. Harborne JB (1973). Phytochemical Methods - A Guide to Modern Techniques of Plant Analysis, Chapman and Hall Ltd, London, pp. 1-279.

131. He W, Wu JJ, Ning J, Hou J, Xin H, He YQ, Ge GB, Xu W (2015). Inhibition of human cytochrome P450 enzymes by licochalcone A, a naturally occurring constituent of licorice. *Toxicol In Vitro*, 29(7): 1569–76.

132. Hellum BH, Hu Z, Nilsen OG (2007). The induction of CYP1A2, CYP2D6 and CYP3A4 by six trade herbal products in cultured primary human hepatocytes. *Basic Clin Pharmacol Toxicol*, 100(1): 23–30.

133. Helwig D (2005). Traditional African medicine. Gale Encyclopedia of Alternative Medicine. Vol 4 (2nd ed.), Thomson Gale Publications, USA.

134. Hewitt NJ, Lecluyse EL, Ferguson, SS (2007). Induction of hepatic cytochrome P450 enzymes: Methods, mechanisms, recommendations, and *in vitro* - *in vivo* correlations. *Xenobiotica*, 37: 1196–1224.

135. Hidaka M, Okumura M, Fujita K, Ogikubo T, Yamasaki K, Iwakiri T, Setoguchi N, Arimori K (2005). Effects of pomegranate juice on human cytochrome P450 3a (Cyp3a) and carbamazepine pharmacokinetics in rats. *Drug Metab Dispos*, 33(5): 644–8.

136. Hillenkamp F, Karas M, Beavis RC, Chait BT (1991). Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal Chem*, 63(24): 1193a–1203a.

137. HIV Clinical Resource (2010). HIV Drug-Drug Interactions - HIV clinical resource, New York State Department of Health (NYSDOH) AIDS Institute (AI), Available from: *https://www.hivguidelines.org*.

138. Ho PC, Saville DJ (2001). Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds. *J Pharm Pharmaceut Sci*, 4(3): 217–227.

139. Hossain MB, Rai DK, Brunton NP, Martin-Diana AB, Barry-Ryan C (2010). Characterization of phenolic composition in Lamiaceae spices by LC-ESI-MS/MS. *J Agric Food Chem*, 58(19): 10576–81.

140. Hu N, Hu M, Duan R, Liu C, Guo H, Zhang M, Yu Y, Wang X, Liu L, Liu X (2014). Increased levels of fatty acids contributed to induction of hepatic CYP3A4 activity induced by diabetes - *in vitro* evidence from HepG2 Cell and Fa2N-4 cell lines. *J Pharmacol Sci*, 124(4): 433–444.

141. Hughes GD, Aboyade OM, Beauclair R, Mbamalu ON, Puoane TR (2015). Characterizing herbal medicine use for noncommunicable diseases in urban South Africa. *Evid Based Complement Alternat Med*, 2015: 736074.

142. Hussain S (2011). Patient counseling about herbal-drug interactions. *Afr J Tradit Complement Altern Med*, 8(5 Suppl): 152–163.

143. Hutzler JM, Wienkers LC, Wahlstrom JL, Carlson TJ, Tracy TS (2003). Activation of cytochrome P450 2C9-mediated metabolism: mechanistic evidence in support of kinetic observations. *Arch Biochem Biophys*, 410(1): 16–24.

144. ICS-UNIDO (2008). Extraction Technologies for medicinal and aromatic Plants. Earth, Environmental and Marine Sciences and Technologies, International Centre for Science and High Technology (ICS-UNIDO), Italy, pp. 1-260. Available at: *https://www.unido.org/sites/default/files/2009-10/Extraction_technologies_for_medicinal_and_aromatic_plants_0.pdf*.

145. Idris OA, Wintola OA, Afolayan AJ (2017). Phytochemical and antioxidant activities of *Rumex crispus* L. in treatment of gastrointestinal helminths in Eastern Cape Province, South Africa. *Asian Pac J Trop Biomed*, 7(12): 1071–1078.

146. Jackson JP, Freeman KM, Friley WW, Herman AG, Black CB, Brouwer KR, Roe AL (2017). Prediction of clinically relevant herb-drug clearance interactions using sandwich-cultured human hepatocytes: *Schisandra* spp. Case Study. *Drug Metab Dispos*, 45(9): 1019–1026.

147. Jadhav BK, Khandelwal KR, Ketkar AR, Pisal SS (2004). Formulation and evaluation of mucoadhesive tablets containing eugenol for the treatment of periodontal diseases. *Drug Dev Ind Pharm*, 30(2): 195–203.

148. Jamis-Dow CA, Katki AG, Collins JM, Klecker RW (1997). Rifampin and rifabutin and their metabolism by human liver esterases. *Xenobiotica*, 27: 1015–24.

149. Jeurissen SM, Claassen FW, Havlik J, Bouwmans EE, Cnubben NH, Sudhölter EJ, Rietjens IM, van Beek TA (2007). Development of an on-line high performance liquid chromatography detection system for human cytochrome P450 1A2 inhibitors in extracts of natural products. *J Chromatogr A*, 1141(1): 81–9.

150. Jia L, Liu X (2007). The conduct of drug metabolism studies considered good practice (II): *in vitro* experiments. *Curr Drug Metab*, 8(8): 822–829.

151. Jin SE, Ha H, Seo CS, Shin HK, Jeong SJ (2018). Expression of hepatic cytochrome P450s in rats administered with Guibi-tang, a traditional herbal formula. *Pharmacogn Mag*, 13(Suppl 4): S822–S827.

152. Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NCO, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT (2000). The pregnane X receptor: A promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol*, 14: 27 – 39.

153. Ju SK, Min HY, Lee EJ, Sam SK (2008). Phytochemical studies on *Astragalus* root (1) - Saponins. *Nat Prod Sci*, 14(1): 37–46.

154. Kalaiarasi P, Pugalendi KV (2009). Antihyperglycemic effect of 18 beta glycyrrhetinic acid, aglycone of glycyrrhizin on streptozotocin diabetic rats. *Eur J Pharmacol*, 1: 269–73.

155. Kang D, Geng T, Lian Y, Li Y, Ding G, Huang W, Ma S, Wang Z, Xiao W (2017). Direct inhibition of Re Du Ning Injection and its active compounds on human liver cytochrome P450 enzymes by a cocktail method. *Biomed Chromatogr*, 31(7).

156. Kar A, Mukherjee P, Saha S, Bahadur S, Ahmmed SKM, Pandit S (2015). Possible herb-drug interaction of *Morus alba* L. - a potential anti-diabetic plant from Indian traditional medicine. *Indian J Trad Med*, 14(4): 626–631.

157. Karakurt S (2016). Modulatory effects of rutin on the expression of cytochrome P450s and antioxidant enzymes in human hepatoma cells. *Acta Pharm*, 66(4): 491–502.

158. Karas M, Krüger R (2003). Ion formation in MALDI: the cluster ionization mechanism. *Chem Rev*, 103(2): 427–440.

159. Karkanis A, Martins N, Petropoulos SA, Ferreira ICFR (2018). Phytochemical composition, health effects, and crop management of liquorice (*Glycyrrhiza glabra* L.):? Medicinal plant. *Food Rev Int*, 34(2): 182–203.

160. Kaul MK, Kumar A, Ahuja A, Mir BA, Suri KA, Qazi GN (2009). Production dynamics of Withaferin A in *Withania somnifera* (L.) Dunal complex. *Nat Prod Res*, 23(14): 1304–11.

161. Kawaguchi S, Nakamura T, Tsuda S, Murashige R, Sasaki YF (2018). Detection of *in vitro* genotoxicity of pro-mutagens using the comet assay under human and rat liver S9 fractions. *MOJ Toxicol*, 4(4): 255–261.

162. Kent UM, Aviram M, Rosenblat M, Hollenberg PF (2002). The liquorice root derived isoflavan glabridin inhibits the activities of human cytochrome P450S 3A4, 2B6, and 2C9. *Drug Metab Dispos*, 30(6): 709–15.

163. Kerantzas CA, Jacobs WR (2017). Origins of combination therapy for Tuberculosis: lessons for future antimicrobial development and application. *mBio*, 8(2): e01586–16.

164. Kivisto KT, Kroemer HK, Eichelbaum M (1995). The role of human cytochrome P450 enzymes in the metabolism of anticancer agents: implications for drug interactions. *Br J Clin Pharmacol*, 40: 523–530.

165. Klieber S, Torreilles F, Guillou F, Fabre G (2010). The use of human hepatocytes to investigate drug metabolism and CYP enzyme induction. *Methods Mol Biol*, 640: 295–308.

166. Kliewer SA, Willson TM (2002). Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J Lipid Res*, 43(3): 359–64.

167. Kobayashi Y, Fukami T, Nakajima A, Watanabe A, Nakajima M, Yokoi T (2012). Species differences in tissue distribution and enzyme activities of arylacetamide deacetylase in human, rat, and mouse. *Drug Metab Dispos*, 40(4): 671–9.

168. Kohl M, Gorden R, Eisenacher M, Schnabel A, Meyer HE, Marcus K, Stephan C (2008). Automated calculation of unique peptide sequences or unambiguous identification of highly homologous proteins by mass spectrometry. *J Proteomics Bioinform*, 1: 6–10.

169. Komoroski BJ, Zhang S, Cai H, Hutzler JM, Frye R, Tracy TS, Strom SC, Lehmann T, Ang CY, Cui YY, Venkataramanan R (2004). Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. *Drug Metab Dispos*, 32(5): 512–8.

170. Kong LM, Xu SY, Hu HH, Zhou H, Jiang HD, Yu LS, Zeng S (2014). Identification of CYP2C19 inhibitors from phytochemicals using the recombinant human enzyme model. *Pharmazie*, 69(5): 362–366.

171. Kopečná-Zapletalová M, Krasulová K, Anzenbacher P, Hodek P, Anzenbacherová E (2017). Interaction of isoflavonoids with human liver microsomal cytochromes P450: inhibition of CYP enzyme activities. *Xenobiotica*, 47(4): 324–331.

172. Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, Tracy TS (1998). Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry*, 37(12): 4137–4147.

173. Kreek MJ, Garfield JW, Gutjahr CL, Giusti LM (1976). Rifampin-induced methadone withdrawal. *N Engl J Med*, 294(20): 1104–6.

174. Kricka LJ (2003). Clinical applications of chemiluminescence. *Analytica Chimica Acta*, 500: 279–286.

175. Křížková J, Burdová K, Stiborová M, Křen V, Hodek P (2009). The effects of selected flavonoids on cytochromes P450 in rat liver and small intestine. *Interdis Tox*, 2(3): 201–204.

176. Kuete V (2017). Medicinal spices and vegetables from Africa (1st ed.) - Therapeutic potential against metabolic, inflammatory, infectious and systemic diseases. Academic Press Elsevier, pp. 694.

177. Kuo WL, Liao HR, Chen JJ (2014). Biflavans, flavonoids, and a dihydrochalcone from the stem wood of *Muntingia calabura* and their inhibitory Activities on neutrophil pro-Inflammatory responses. Molecules, 19: 20521–20535.

178. Kurapati KR, Atluri VS, Samikkannu T, Nair MP (2013). *Ashwagandha* (*Withania somnifera*) reverses β-amyloid1-42 induced toxicity in human neuronal cells: implications in HIV-associated neurocognitive disorders (HAND). *PLoS ONE*, 8(10): e77624.

179. Kwara A, Ramachandran G, Swaminathan S (2010). Dose adjustment of the non-nucleoside reverse transcriptase inhibitors during concurrent rifampicin-containing tuberculosis therapy: one size does not fit all. *Expert Opin Drug Metab Toxicol*, 6(1): 55–68.

180. Kyokawa Y, Nishibe Y, Wakabayashi M, Harauchi T, Maruyama T, Baba T, Ohno K (2001). Induction of intestinal cytochrome P450 (CYP3A) by rifampicin in beagle dogs. *Chem Biol Interact*, 134(3): 291–305.

181. Lahoz A, Gombau L, Donato MT, Castell JV, Gomez-Lechon MJ (2006). *In vitro* ADME medium/high-throughput screening in drug preclinical development. *Mini Rev Med Chem*, 6(9): 1053–1062.

182. Lai PK, Roy J (2004). Antimicrobial and chemopreventive properties of herbs and spices. *Curr Med Chem*, 11(11): 1451–60.

183. Lau C, Mooiman KD, Maas-Bakker RF, Beijnen JH, Schellens JH, Meijerman I (2013). Effect of Chinese herbs on CYP3A4 activity and expression *in vitro*. *J Ethnopharmacol*, 149(2): 543–9.

184. LeCluyse E, Madan A, Hamilton G, Carroll K, DeHaan R, Parkinson A (2000). Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. *J Biochem Mol Toxicol*, 14: 177–178.

185. Lee EJ, Min-Hye Y, Hye-Sil J, Ju-Sun K, Sam-Sik K (2008). Phytochemical studies on Astragalus root (2); flavonoids and a lignan. *Nat Prod Sci*, 14(2): 131–137.

186. Lee SY, Jang H, Lee JY, Kwon KI, Oh SJ, Kim SK (2014). Inhibition of cytochrome P450 by ethambutol in human liver microsomes. *Toxicol Lett*, 229(1): 33–40.

187. Leyden JJ, Shergill B, Micali G, Downie J, Wallo W (2011). Natural options for the management of hyperpigmentation. *J Eur Acad Dermatol Venereol*, 25(10): 1140–5.

188. Li G, Simmler C, Chen L, Nikolic D, Chen SN, Pauli GF, van Breemen RB (2017). Cytochrome P450 inhibition by three licorice species and fourteen licorice constituents. *Eur J Pharm Sci*, 109: 182–190.

189. Li L, Liang S, Du F, Li C (2007). Simultaneous quantification of multiple *Licorice* flavonoids in rat plasma. *J Am Soc Mass Spectrom*, 18: 778–782.

190. Li M, Hou XF, Zhang J, Wang SC, Fu Q, He LC (2011). Applications of HPLC/MS in the analysis of traditional Chinese medicines. *J Pharma Anal*, 1(2): 81–91.

191. Li S, Lin Z, Jiang H, Tong L, Wang H, Chen S (2016). Rapid Identification and Assignation of the active ingredients in *Fufang banbianlian* injection using HPLC-DAD-ESI-IT-TOF-MS. *J Chromatogr Sci*, 54(7): 1225–37.

192. Lin JH (2006). CYP induction - mediated drug interactions: *In vitro* assessment and clinical implications. *Pharm Res*, 23(6): 1089–1116.

193. Liu Y, Liu J, Wang Y, Abozeid A, Tang ZH (2016). Simultaneous determination of six active metabolites in *Astragalus mongholicus* (Fisch.) Bge. under salt stress by ultra-pressure liquid chromatography with tandem mass spectrometry. *Springerplus*, 5(1): 927.

194. Liu Y, Liu J, Wu KX, Guo XR, Tang ZH (2018). A rapid method for sensitive profiling of bioactive triterpene and flavonoid from *Astragalus mongholicus* and *Astragalus membranaceus* by ultra-pressure liquid chromatography with tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, 1085: 110–118.

195. Loughrin JH, Kasperbauer MJ (2003). Aroma content of fresh basil (*Ocimum basilicum* L.) leaves is affected by light reflected from colored mulches. *J Agric Food Chem*, 51(8): 2272–6.

196. Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E, Gan LS (2002). CYP3A4 induction by drugs: Correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos*, 30: 795–804.

197. Machado BAS, Silva RPD, Barreto G de A, Costa SS, da Silva DF, Brandão HN, da Rocha JLC, Dellagostin OA, Henriques JAP, Umsza-Guez MA, Padilha FF (2016). Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red *Propolis* derived from different geographic regions in Brazil. *PLoS One*, 11(1): e0145954.

198. Madgula VL, Avula B, Pawar RS, Shukla YJ, Khan IA, Walker LA, Khan SI (2010). Characterization of *in vitro* pharmacokinetic properties of hoodigogenin A from *Hoodia gordonii*. *Planta Med*, 76(1): 62–9.

199. Mahdi M, Szojka Z, Mótyán JA, Tőzsér J (2015). Inhibition profiling of retroviral protease inhibitors using an HIV-2 modular system. *Viruses*, 7(12): 6152–62.

200. Mahomoodally MF (2013). Traditional medicines in Africa: An appraisal of ten potent African medicinal plants. *Evid Based Complement Alternat Med*, 2013: 617459.

201. Mallick P, Schirle M, Chen SS, Flory MR, Lee H, Martin D, Ranish J, Raught B, Schmitt R, Werner T, Kuster B, Aebersold R (2007). Computational prediction of proteotypic peptides for quantitative proteomics. *Nat Biotechnol*, 25: 125–131.

202. Mammen JS, Kleiner HE, DiGiovanni J, Sutter TR, Strickland PT (2005). Coumarins are competitive inhibitors of cytochrome P450 1B1, with equal potency for allelic variants. *Pharmaco Geno*, 15: 183–188.

203. Mandery K, Bujok K, Schmidt I, Keiser M, Siegmund W, Balk B, König J, Fromm MF, Glaeser H (2010). Influence of the flavonoids apigenin, kaempferol, and quercetin on the function of organic anion transporting polypeptides 1A2 and 2B1. *Biochem Pharmacol*, 80(11): 1746–53.

204. Manosroi J, Dhumtanom P, Manosroi A (2006). Anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines. *Cancer Lett*, 235(1): 114–20.

205. Manosuthi W, Wiboonchutikul S, Sungkanuparph S (2016). Integrated therapy for HIV and tuberculosis. *AIDS Res Ther*, 13: 22.

206. Martikainen L (2012). *In Vitro* and *in silico* methods to predict cytochrome P450 enzyme inhibition. Dissertations in Health Sciences, University of Eastern Finland, 108p.

207. Masevhe NA, McGaw LJ, Eloff JN (2015). The traditional use of plants to manage candidiasis and related infections in Venda, South Africa. *J Ethnopharmacol*, 168: 364–72.

208. Masimirembwa CM, Bredberg U, Andersson TB (2003). Metabolic stability for drug discovery and development: pharmacokinetic and biochemical challenges. *Clin Pharmacokin*, 42: 515–528.

209. Mazumdar PM, Patnayak SP, Parwani H (2012). Evaluation of immunomodulatory activity of *Glycyrrhiza glabra* L roots in combination with zing. *Asian Pac J Trop*, s15–s20.

210. Mehta P, Dhapte V (2015). Cupping therapy: A prudent remedy for a plethora of medical ailments. *J Tradit Complement Med*, 5(3): 127–134.

211. Meintjes G, Moorhouse MA, Carmona S, Davies N, Dlamini S, van Vuuren C, Manzini T, Mathe M, Moosa Y, Nash J, Nel J, Pakade Y, Woods J, Van Zyl G, Conradie F, Venter F (2017). Adult antiretroviral therapy guidelines 2017. *South Afr J HIV Med*, 18(1), 776.

212. Mena P, Calani L, Dall'Asta C, Galaverna G, García-Viguera C, Bruni R, Crozier A, Rio DD (2012). Rapid and comprehensive evaluation of (Poly) phenolic compounds in pomegranate (*Punica granatum* L.) juice by UHPLC-MS^n. *Molecules*, 17: 14821–14840.

213. Messier C, Epifano F, Genovese S, Grenier D (2012). Liquorice and its potential beneficial effects in common oro-dental diseases. *Oral Dis*, 18(1): 32–9.

214. Michaels S, Wang MZ (2014). The revised human liver cytochrome P450 "Pie": absolute protein quantification of CYP4F and CYP3A enzymes using targeted quantitative proteomics. *Drug Metab Dispos*, 42(8): 1241–51.

215. Mills E, Cooper C, Seely D, Kanfer I (2005). African herbal medicines in the treatment of HIV: *Hypoxis* and *Sutherlandia*. An overview of evidence and pharmacology. *Nutr J*, 4: 19.

216. Mills JB, Rose KA, Sadagopan N, Sahi J, de Morais SM (2004). Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. *J Pharmacol Exp Ther*, 309: 303–309.

217. Ming LJ, Yin AC (2013). Therapeutic effects of glycyrrhizic acid. *Nat Prod Commun*, 8(3): 415–8.

218. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J (2009). Steroidal lactones from Withania somnifera, an ancient plant for novel medicine. *Molecules*, 14(7): 2373–2393.

219. Misaka S, Kawabe K, Onoue S, Werba JP, Giroli M, Tamaki S, Kan T, Kimura J, Watanabe H, Yamada S (2013). Effects of green tea catechins on cytochrome P450 2B6, 2C8, 2C19, 2D6 and 3A activities in human liver and intestinal microsomes. *Drug Metab Pharmacokinet*, 28(3): 244–249.

220. Modarai M, Gertsch J, Suter A, Heinrich M, Kortenkamp A (2007). Cytochrome P450 inhibitory action of Echinacea preparations differs widely and co-varies with alkylamide content. *J Pharm Pharmacol*, 59: 567–573.

221. Modarai M, Gertsch J, Suter A, Heinrich M, Kortenkamp A (2011). The interaction potential of herbal medicinal products: a luminescence-based screening platform assessing effects on cytochrome P450 and its use with devil's claw (*Harpagophytiradix*) preparations. *J Pharm Pharmacol*, 63(3): 429–38.

222. Mohan B, Sharda N, Singh S (2003). Evaluation of the recently reported USP gradient HPLC method for analysis of anti-tuberculosis drugs for its ability to resolve degradation products of rifampicin. *J Pharm Biomed Anal*, 31(3): 607–612.

223. Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, Kliewer SA (2000). Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem*, 275: 15122–15127.

224. Mueller SC, Majcher-Peszynska J, Uehleke B (2006). The extent of induction of CYP3A by St. John's wort varies among products and is linked to hyperforin dose. *Eur J Clin Pharmacol*, 62(1): 29–36.

225. Mulata HN, Gnanasekaran N, Melaku U, Daniel S (2015). Phytochemical Screening and Assessment of *In Vitro* Antioxidant Activities of *Calpurnia Aurea* Seeds and Leaves. *IJPPR*, 2(2): 1–12.

226. Müllertz A, Perrie Y, Rades T (2016). Analytical techniques in the pharmaceutical sciences. Springer, New York, NY, ISBN 978-1-4939-4027-1, ISSN 2192-6204.

227. Murphy E, Buckley B, Lippincott L, Yang I, Rosen B (2003). The characterization of tentatively identified compounds (TICs) in Samples from Public Water Systems in New Jersey (Report). New Jersey Department of Environmental Protection Division of Science, Research & Technology. Available at: *https://www.state.nj.us/dep/dsr/TIC-report.pdf*.

228. Muto S, Fujita KI, Yamazaki Y, Kamataki T (2001). Inhibition by green tea catechins of metabolic activation of procarcinogens by human cytochrome P450. *Mutat Res*, 479(1): 197–206.

229. Nagarajappa SH, Pandit S, Divanji M, Mariyanna B, Kumar P, Godavarthi A (2016). Effect of *Coleus forskohlii* and its major constituents on cytochrome P450 induction. *J Tradit Complement Med*, 6(1): 130–133.

230. Nakajima M, Yoshida R, Shimada N, Yamazaki H, Yokoi T (2001). Inhibition and inactivation of human cytochrome P450 isoforms by phenethyl isothiocyanate. *Drug Metab Dispos*, 29(8): 1110–3.

231. Naritomi Y, Terashita S, Kagayama A (2004). Identification and relative contributions of human cytochrome P450 isoforms involved in the metabolism of glibenclamide and lansoprazole: evaluation of an approach based on the *in vitro* substrate disappearance rate. *Xenobiotica*, 34(5): 415–427.

232. NCCAM-NIH (2006). A Closer Look at Ayurvedic Medicine. Focus on complementary and alternative medicine (Bethesda, Maryland: National Center for Complementary and Alternative Medicine (NCCAM), US National Institutes of Health (NIH)), 12(4).

233. NCCAM-NIH (2010). Herbs at a glance - A quick guide to herbal supplements. National Center for Complementary and Alternative Medicine (NCCAM), US National Institutes of Health (NIH), PubNo. 10-6248. Available at: *https://www.northmiresouces.org/uploads/5/2/7/7/5277004/herbs_at_a_glance.__a_quick_guide_to_herbal_supplements_may_2011.pdf*.

234. Nebert DW, Russell DW (2002). Clinical importance of the cytochromes P450. *Lancet*, 360: 1155–1162.

235. Nelson DL, Cox MM (2008). Lehninger Principles of Biochemistry (5th ed.), Freeman WH and Company, USA.

236. Ngaimisi E, Habtewold A, Minzi O, Makonnen E, Mugusi S, Amogne W, Yimer G, Riedel KD, Janabi M, Aderaye G, Mugusi F, Bertilsson L, Aklillu E, Burhenne J (2013). Importance of ethnicity, CYP2B6 and ABCB1 genotype for efavirenz pharmacokinetics and

treatment outcomes: A parallel-group prospective cohort study in two Sub-Saharan Africa populations. *PLoS One*, 8(7): e67946.

237. Nguyen S, Huang H, Foster BC, Tam TW, Xing T, Smith ML, Arnason JT, Akhtar H (2014). Antimicrobial and P450 inhibitory properties of common functional foods. *J Pharm Pharm Sci*, 17(2): 254–265.

238. Nilsen BM, Berg K, Goksøyr A (1998). Induction of Cytochrome P450 1A (CYP1A) in fish. A biomarker for environmental pollution. *Methods Mol Biol*, 107: 423–438.

239. Nomeir AA, Palamanda JR, Favreau L (2004). Identification of CYP mechanism-based inhibitors. In: Yan Z, Caldwell GW (eds) Optimization in Drug Discovery. Methods in Pharmacology and Toxicology. Humana Press, USA, pp. 245–262.

240. Ntie-Kang F, Lifongo LL, Mbaze LM, Ekwelle N, Owono LCO, Megnassan E, N Judson PN, Wolfgang Sippl W, Efange SMN (2013). *In silico* drug metabolism and pharmacokinetic profiles of natural products from medicinal plants in the Congo basin. *BMC Complement Altern Med*, 13: 147.

241. Ochs RS (2000). Understanding enzyme inhibition. *J Chem Educ*, 77(11): 1453.

242. Ogburn ET, Jones DR, Masters AR, Xu C, Guo Y, Desta Z (2010). Efavirenz primary and secondary metabolism *in vitro* and *in vivo*: identification of novel metabolic pathways and cytochrome P450 (CYP) 2A6 as the principal catalyst of efavirenz 7-hydroxylation. *Drug Metab Dispos*, 38(7): 1218–29.

243. Ogu CC, Maxa JL (2000). Drug interactions due to cytochrome P450. *Proc (Bayl Univ Med Cent)*, 13(4): 421–423.

244. Onwuanibe RC (1979). The philosophy of African medical practice. *A J Opinion JSTOR*, 9(3): 25–28.

245. Pan Y, Tiong KH, Abd-Rashid BA, Ismail Z, Ismail R, Mak JW, Ong CE (2014). *In vitro* effect of important herbal active constituents on human cytochrome P450 1A2 (CYP1A2) activity. *Phytomedicine*, 21(12): 1645–1650.

246. Panchagnula R, Sood A, Sharda N, Kaur K, Kaul CL (1999). Determination of rifampicin and its main metabolite in plasma and urine in presence of pyrazinamide and isoniazid by HPLC method. *J Pharm Biomed Anal*, 18(6): 1013–20.

247. Pandey A, Tripathi S (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *J Pharmacogn Phytochem*, 2(5): 115–119.

248. Pandit S, Ponnusankar S, Bandyopadhyay A, Ota S, Mukherjee PK (2011). Exploring the possible metabolism mediated interaction of *Glycyrrhiza glabra* extract with CYP3A4 and CYP2D6. *Phytother Res*, 25(10): 1429–34.

249. Panel on antiretroviral guidelines for adults and adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. Accessed [14 Oct 2017].Available from: *http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf*.

250. Pao LH, Hu OY, Fan HY, Lin CC, Liu LC, Huang PW (2012). Herb-drug interaction of 50 Chinese herbal medicines on CYP3A4 activity *in vitro* and *in vivo*. *Am J Chin Med*, 40(1): 57–73.

251. Paolini M, Pozzetti L, Sapone A, Cantelli-Forti G (1998). Effect of licorice and glycyrrhizin on murine liver CYP-dependent monooxygenases. *Life Sci*, 62(6): 571–582.

252. Park D, Jeon JH, Shin S, Joo SS, Kang DH, Moon SH, Jang MJ, Cho YM, Kim JW, Ji HJ, Ahn B, Oh KW, Kim YB (2009). Green tea extract increases cyclophosphamide induced teratogenesis by modulating the expression of cytochrome P-450 mRNA. *Reprod Toxicol*, 27(1): 79–84.

253. Patel JA, Anderson E, Dong J (2009). False Positive Ultrasensitive HIV bDNA Viral Load Results in Diagnosis of Perinatal HIV-Infection in the era of low transmission. *Lab Med*, 40(10): 611–614.

254. Pawar RS, Shukla YJ, Khan SI, Avula B, Khan IA (2007). New oxypregnane glycosides from appetite suppressant herbal supplement *Hoodia gordonii*. *Steroids*, 72(6-7): 524–534.

255. Payyappallimana U, Subramanian SM (2015). Traditional medicine in connecting global priorities: biodiversity and human health: a state of knowledge review. In: C. Romanelli, et al.(eds.) World Health Organisation/Secretariat of the UN Convention on Biological Diversity: Geneva, pp. 180–199.

256. Pereira CA, Silva Pereira LL, Corrêa AD (2010). *Hoodia gordonii* in the treatment of obesity: A review. *J Med Plant Res*, 4(22): 2305–2312.

257. Petkova NT, Vrancheva RZ, Mihaylova DS, Ivanov I, Pavlov A, Denev PP (2015). Antioxidant activity and fructan content in root extracts from *elecampane* (*Inula helenium* L.). *J BioSci Biotechnol*, 4(1): 101–107.

258. PharmVar (2017). Pharmacogene Variation Consortium The human cytochrome P450 (CYP) allele nomenclature database. Available from: *https://www.pharmvar.org/*.

259. Pimkaew P, Küblbeck J, Petsalo A, Jukka J, Suksamrarn A, Juvonen R, Auriola S, Piyachaturawat P, Honkakoski P (2013). Interactions of sesquiterpenes zederone and germacrone with the human cytochrome P450 system. *Toxicol In Vitro*, 27(6): 2005–2012.

260. Pirbalouti AG, Mohammadi M (2013). Phytochemical composition of the essential oil of different populations of *Stachys lavandulifolia* Vahl. *Asian Pac J Trop Biomed*, 3(2): 123–128.

261. Pohl C, Will F, Dietrich H, Schrenk D (2006). Cytochrome P450 1A1 expression and activity in Caco-2 cells: modulation by apple juice extract and certain apple polyphenols. *J Agric Food Chem*, 54(26): 10262–10268.

262. Polasek TM, Elliot DJ, Somogyi AA, Gillam EMJ, Lewis BC, Miners JO (2006). An evaluation of potential mechanism-based inactivation of human drug metabolizing cytochromes P450 by monoamine oxidase inhibitors, including isoniazid. *Br J Clin Pharmacol*, 61(5): 570–584.

263. Polsky-Fisher SL, Cao H, Lu P, Gibson CR (2006).Effect of cytochromes P450 chemical inhibitors and monoclonal antibodies on human liver microsomal esterase activity. *Drug Metab Dispos*, 34(8): 1361–6.

264. Powell M, D'Arcy MB (2013). Liquid-phase separation of structurally similar steroids using phenyl stationary phases. *Anal Methods*, 5: 5014–5018.

265. Prakash J, Velpandian T, Pande JN, Gupta SK (2003). Serum rifampicin levels in patients with tuberculosis: effect of P-glycoprotein and CYP3A4 blockers on its absorption. *Clin Drug Investig*, 23: 463–72.

266. Prevoo D, Smith C, Swart P, Swart AC (2004). The effect of *Sutherlandia frutescens*on steroidogenesis: confirming indigenous wisdom. *Endocr Res*, 30: 745–751.

267. Pu QH, Shi L, Yu C (2015). Time-dependent inhibition of CYP3A4 by gallic acid in human liver microsomes and recombinant systems. *Xenobiotica*, 45(3): 213–7.

268. Punyasiri PAN, Jeganathan B, Dananjaya Kottawa-Arachchi J, Ranatunga MAB, Abeysinghe ISB, Gunasekare MTK, Bandara BMR (2015). New Sample Preparation Method for quantification of phenolic compounds of tea (*Camellia sinensis* L. Kuntze): A polyphenol rich plant. *J Anal Methods Chem*, 964341.

269. Quintieri L, Palatini P, Nassi A, Ruzza P, Floreani M (2008). Flavonoids diosmetin and luteolin inhibit midazolam metabolism by human liver microsomes and recombinant CYP 3A4 and CYP3A5 enzymes. *Biochem Pharmacol*, 75(6): 1426–1437.

270. Qin CZ, Lv QL, Wu NY, Cheng L, Chu YC, Chu TY, Hu L, Cheng Y, Zhang X, Zhou HH (2015). Mechanism-based inhibition of Alantolactone on human cytochrome P450 3A4 *in vitro* and activity of hepatic cytochrome P450 in mice. *J Ethnopharmacol*, 168: 146–9.

271. Rachel P (2006). "What's that stuff? Marshmallow". *Chem Eng News*, 84(16): 41.

272. Raaman N (2006). Phytochemical Technique. New Indian Publishing Agencies, New Delhi, 19, ISBN 10: 8189422308, ISBN 13: 9788189422301.

273. Ramos RTM, Bezerra ICF, Ferreira MRA, Soares LAL (2017). Spectrophotometric Quantification of Flavonoids in Herbal Material, Crude Extract, and Fractions from Leaves of *Eugenia uniflora* Linn. *Pharmacognosy Res*, 9(3): 253–260.

274. Ramsay RR, Tipton KF (2017). Assessment of enzyme inhibition: A review with examples from the development of monoamine oxidase and cholinesterase inhibitory drugs. *Molecules*, 22: 1192.

275. Rani S, Khan SA, Ali M (2010). Phytochemical investigation of the seeds of *Althaea officinalis* L. *Nat Prod Res*, 24(14): 1358–64.

276. Reddy YP, Chandrasekhar KB, Sadiq MJ (2015). A study of *Nigella sativa* induced growth inhibition of MCF and HepG2 cell lines: An anti-neoplastic study along with its mechanism of action. *Pharmacognosy Res*, 7(2): 193–197.

277. Rege A, Ambaye RY, Deshmukh RA (2010). *In vitro* testing of anti-hiv activity of some medicinal plants. *Indian J Natural Prod Resour*, 1(2): 193–199.

278. Reuter J, Merfort I, Schempp CM (2010). Botanicals in dermatology: an evidence-based review. *Am J Clin Dermatol*, 11(4): 247–67.

279. Roy JN, Lajoie J, Zijenah LS, Barama A, Poirier C, Ward BJ, Roger M (2005). CYP3A5 genetic polymorphisms in different ethnic populations. *Drug Metab Dispos*, 33(7): 884–7.

280. Said RB, Hamed AI, Mahalel UA, Al-Ayed AS, Kowalczyk M, Moldoch J, Oleszek W, Stochmal A (2017). Tentative characterization of polyphenolic compounds in the male flowers of *Phoenix dactylifera* by liquid chromatography coupled with mass spectrometry and DFT. *Int J Mol Sci*, 18: 512.

281. Salphati L, Benet LZ (1998). Effects of ketoconazole on digoxin absorption and disposition in rat. *Pharmacology*, 56: 308–313.

282. Sato H, Goto W, Yamamura J, Kurokawa M, Kageyama S, Takahara T, Watanabe A, Shiraki K (1996). Therapeutic basis of glycyrrhizin on chronic hepatitis B. *Antiviral Res*, 30(2-3): 171–7.

283. Savai J, Varghese A, Pandita N, Chintamaneni M (2015). Investigation of CYP3A4 and CYP2D6 Interactions of *Withania somnifera* and *Centella asiatica* in human liver microsomes. *Phytother Res*, 29(5): 785–90.

284. Savai J, Pandita N, Chintamaneni M (2014). Investigation of CYP1A interaction potential of *Withania Somnifera* in rat and human liver microsomes. *Indian J Pharm Pharma Sci*, 76(2): 138–147.

285. Savai J, Varghese A, Pandita NP (2013). Lack of the cytochrome P450 3A interaction of methanolic extract of Withania somnifera, Withaferin A, Withanolide A and Withanoside IV. *J Pharm Negat Results*, 4(1): 26–32.

286. Scartezzini P, Speroni E (2000). Review on some plants of Indian traditional medicine with antioxidant activity. *J Ethnopharmacol*, 71(1-2): 23–43.

287. Schipani A, Wyen C, Mahungu T, Hendra H, Egan D, Siccardi M, Gerry D, Saye K, Gerd F, Michael Y, Jürgen R, Norbert BH, Margaret JA, Andrew O, David BJ (2011). Integration of population pharmacokinetics and pharmacogenetics: an aid to optimal nevirapine dose selection in HIV-infected individuals. *J Antimicrob Chemother*, 66(6): 1332–1339.

288. Seca AM, Grigore A, Pinto DC, Silva AM (2014). The genus *Inula* and their metabolites: from ethnopharmacological to medicinal uses. *J Ethnopharmacol*, 154(2): 286–310.

289. Sendker J, Böker I, Lengers I, Brandt S, Jose J, Stark T, Hofmann T, Fink C, Abdel-Aziz H, Hensel A (2017). Phytochemical characterization of low molecular weight constituents from *marshmallow* roots (*Althaea officinalis*) and inhibiting effects of the aqueous extract on human hyaluronidase-1. *J Nat Prod*, 80: 290–297.

290. Seng KY, Hee KH, Soon GH, Chew N, Khoo SH, Lee LS (2015). Population pharmacokinetics of rifampicin and 25-deacetyl-rifampicin in healthy Asian adults, *J Antimicrob Chemother*, 70(12): 3298–306.

291. Shan W, Zhang Y, Zhu J, Shao Q, Fan X (2012). Inhibitory effects of astragaloside IV on cytochrome P450 enzyme of rat liver microsomes [Article in Chinese]. *Zhongguo Zhong Yao Za Zhi*, 37(1): 85–8.

292. Shou M, Dai R, Cui D, Korzekwa KR, Baillie TA, Rushmore TH (2001). A kinetic model for the metabolic interaction of two substrates at the active site of cytochrome P450 3A4. *The J Biol Chem*, 276(3): 2256–62.

293. Showman AF, Baker JD, Linares C, Naeole CK, Borris R, Johnston E, Konanui J, Turner H (2015). Contemporary pacific and western perspectives on `awa (*Piper methysticum*) toxicology. *Fitoterapia*, 100: 56–67.

294. Shukla SJ, Nguyen DT, MacArthur R, Simeonov A, Frazee WJ, Hallis TM, Marks BD, Singh U, Eliason HC, Printen J, Austin CP, Inglese J, Auld DS (2009). Identification of pregnane X receptor ligands using time - resolved fluorescence resonance energy transfer and quantitative high – throughput screening. *Assay Drug Dev Technol*, 7: 143–159.

295. Shukla YJ, Pawar RS, Ding Y, Li XC, Ferreira D, Khan IA (2009). Pregnane glycosides from *Hoodia gordonii*. *Phytochemistry*, 70(5): 675–683.

296. Si D, Wang Y, Zhou YH, Guo Y, Wang J, Zhou H, Li ZS, Fawcett JP (2009). Mechanism of CYP2C9 inhibition by flavones and flavonols. *Drug Metab Dispos*, 37(3): 629–34.

297. Siddhuraju P, Becker K (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *J Agric Food Chem*, 51(8): 2144–55.

298. Siddiqui BS, Bhatti HA, Begum S, Perwaiz S (2012). Evaluation of the antimycobacterium activity of the constituents from *Ocimum basilicum* against *Mycobacterium tuberculosis*. *J Ethnopharmacol*, 144(1): 220–2.

299. Simirgiotis MJ, Benites J, Areche C, Sepúlveda B (2015). Antioxidant capacities and analysis of phenolic compounds in three endemic *Nolana* species by HPLC-PDA-ESI-MS. *Molecules*, 20: 11490–11507.

300. Simonetti G (1990). Stanley Schuler, ed. Simon & Schuster's Guide to Herbs and Spices. Simon & Schuster, Inc., New York, USA, 256p, ISBN-10: 067173489X, ISBN-13: 978-0671734893.

301. Singer J, Adams J (2014). Integrating complementary and alternative medicine into mainstream healthcare services: the perspectives of health service managers. *BMC Complement Altern Med*, 14: 167.

302. Slaughter RL, Edwards DJ (1995). Recent advances: the cytochrome P450 enzymes. *Ann Pharmacother*, 29: 619–24.

303. Soleimany V, Banaee M, Mohiseni M, Hagi NB, Dehmourdi ML (2016). Evaluation of pre-clinical safety and toxicology of *Althaea officinalis* extracts as naturopathic medicine for common carp (*Cyprinus carpio*). *IJFS*, 15(2): 613–629.

304. Son JS, Chang YJ, Choi YD, Kim SU (1998). Role of jasmonic acid in biotransformation of (--)-isopiperitenone in suspension cell culture of *Mentha piperita*. *Mol Cells*, 8(3): 366–9.

305. Song SH, Chang HE, Jun SH, Park KU, Lee JH, Lee EM, Song YH, Song J (2013). Relationship between CES2 genetic variations and rifampicin metabolism. *J Antimicrob Chemother*, 68(6): 1281–4.

306. Soule JA (2011). Father Kino's Herbs: Growing and using them today, Tierra del Sol Institute Press, Tucson, AZ, USA.

307. Špičáková A, Szotáková B, Dimunová D, Myslivečková Z, Kubíček V, Ambrož M, Lněničková K, Krasulová K, Anzenbacher P, Skálová L (2017). Nerolidol and farnesol inhibit some cytochrome P450 activities but did not affect other xenobiotic-metabolizing enzymes in rat and human hepatic subcellular fractions. *Molecules*, 22, 509.

308. Sridhar A, Sandeep Y, Krishnakishore C, Sriramnaveen P, Manjusha Y, Sivakumar V (2012). Fatal poisoning by isoniazid and rifampicin. *Indian J Nephrol*, 22(5): 385–387.

309. Stanley B (2004). 'Recognition and respect for African traditional medicine'. Canada's International Development Research Centre, CN. Available at: *https://www.idrc.ca/en/article/recognition-and-respect-african-traditional-medicine*.

310. SOTWP (2017). State of the world's plants report 2017, Royal Botanic Gardens Kew, Sfumato foundation, UK. Available from:*https://stateoftheworldsplants.com/2017/report/SOTWP_2017.pdf*.

311. Stander MA, Van Wyk BE, Taylor MJC, Long HS (2017). Analysis of phenolic compounds in rooibos tea (*Aspalathus linearis*) with a comparison of flavonoid-based compounds in natural populations of plants from different regions. *J Agric Food Chem*, 65(47): 10270–10281.

312. Staudinger JL, Chenshu X, Cui YJ, Klaasen CD (2010). Nuclear receptor-mediated regulation of carboxylesterase expression and activity. *Expert Opin Drug Metab Toxicol*, 6: 261–271.

313. Steuck M, Hellhake S, Schebb NH (2016). Food polyphenol apigenin inhibits the cytochrome P450 monoxygenase branch of the arachidonic acid cascade. *J Agric Food Chem*, 64(47): 8973–8976.

314. Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M (1999). The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem*, 274(10): 6043–6.

315. Sugatani J, Sadamitsu S, Wada T, Yamazaki Y, Ikari A, Miwa M (2012). Effects of dietary inulin, statin, and their co-treatment on hyperlipidemia, hepatic steatosis and changes in drug-metabolizing enzymes in rats fed a high-fat and high-sucrose diet. *Nutr Metab (Lond)*, 9(1): 23.

316. Sun DX, Lu JC, Fang ZZ, Zhang YY, Cao YF, Mao YX, Zhu LL, Yin J, Yang L (2010). Reversible inhibition of three important human liver cytochrome p450 enzymes by tiliroside. *Phytother Res*, 24(11): 1670–5.

317. Sutovska M, Nosalova G, Franova S, Kardosova A (2007). The antitussive activity of polysaccharides from *Althaea officinalis* l., var. Robusta, *Arctium lappa* L., var. Herkules, and *Prunus persica* L. *Batsch Bratisl Lek Listy*, 108(2): 93–9.

318. Tang C, Lin JH, Lu AY (2005). Metabolism-based drug–drug interactions: what determines individual variability in cytochrome P450 induction? *Drug Metab Dispos*, 33: 603–613.

319. Tang D, Chen K, Huang L, Li J (2017). Pharmacokinetic properties and drug interactions of apigenin, a natural flavone. *Expert Opin Drug Metab Toxicol*, 13(3): 323–330.

320. Tapsell LC, Hemphill I, Cobiac L, Patch CS, Sullivan DR, Fenech M, Roodenrys S, Keogh JB, Clifton PM, Williams PG, Fazio VA, Inge KE (2006). Health benefits of herbs and spices: the past, the present, the future. *Med J Aust*, 185(4 Suppl): s4–s24.

321. Thakur D, Jain A, Ghoshal G (2016). Evaluation of phytochemical, antioxidant and antimicrobial properties of glycyrrhizin extracted from roots of Glycyrrhiza Glabra. *JSIR* 75(08): 487–494.

322. Thapliyal R, Maru GB (2001). Inhibition of cytochrome P450 isozymes by curcumins *in vitro* and *in vivo*. *Food Chem Toxicol*, 39(6): 541–7.

323. The Royal Society (2005). Personalised medicines: hopes and realities (Report). The Royal Society, London, pp. 1-56. Available at: *https://royalsociety.org/~/media/royal_society_content/policy/publications/2005/9631.pdf*.

324. Thelingwani RS, Dhansay K, Smith P, Chibale K, Masimirembwa CM (2012). Potent inhibition of CYP1A2 by Frutinone A, an active ingredient of the broad spectrum antimicrobial herbal extract from *P. fruticosa*. *Xenobiotica*, 42(10): 989–1000.

325. Thomford NE, Awortwe C, Dzobo K, Adu F, Chopera D, Wonkam A, Skelton M, Blackhurst D, Dandara C (2016). Inhibition of CYP2B6 by medicinal plant extracts: implication for use of efavirenz and nevirapine-based highly active anti-retroviral therapy (HAART) in resource-limited settings. *Molecules*, 21: 211.

326. Thring TSA, Weitz FM (2006). Medicinal plant use in the Bredasdorp/ Elim region of the Southern Overberg in the Western Cape Province of South Africa. *J Ethnopharmacol*, 103(2): 261–75.

327. Tihanyi K, Vastag M (2011). Solubility, delivery and ADME problems of drugs and drug-candidates, Bentham Science Publishers, UAE, ISBN: 978-1-60805-619-4, DOI: 10.2174/97816080512051110101, pp. 1-232.

328. Tirona RG, Leake BF, Wolkoff AW, Kim RB (2003). Human organic anion transporting polypeptide-C (SLC21A6) is a major determinant of rifampin-mediated pregnane X receptor activation. *J Pharmacol Exp Ther*, 304(1): 223–8.

329. Tracy TS (2006). Atypical cytochrome p450 kinetics: implications for drug discovery. *Drugs R D*, 7: 349–363.

330. Tu JH, He YJ, Chen Y, Fan L, Zhang W, Tan ZR, Huang YF, Guo D, Hu DL, Wang D, Zhou HH (2010). Effect of glycyrrhizin on the activity of CYP3A enzyme in humans. *Eur J Clin Pharmacol*, 66(8): 805–810.

331. Turpeinen M, Nieminen R, Juntunen T, Taavitsainen P, Raunio H, Pelkonen O (2004). Selective inhibition of CYP2B6-catalyzed bupropion hydroxylation in human liver microsomes *in vitro*. *Drug Metab Dispos*, 32(6): 626–31.

332. Ubani LU (2011). Preventive Therapy in Complimentary Medicine, Xlibris Corporation, USA, ISBN: 9781462876860, pp. 1-768.

333. Ugochukwu SC, Uche AI, Ifeanyi O (2013).Preliminary phytochemical screening of different solvent extracts of stem bark and roots of *Dennetia tripetala* G. Baker. *Asian J Plant Sci Res*, 2013, 3(3): 10–13.

334. United Press Int (2009). Herbal chemical helps combat HIV – United Press International, USA. Retrieved January 28, 2011. Available at: *https://www.upi.com/Health_News/2009/01/01/Herbal_chemical_helps_combat_HIV/UPI-75921230846928/*.

335. Urdea MS, Horn T, Fultz TJ, Anderson M, Running JA, Hamren S, Ahle D, Chang CA (1991). Branch DNA amplification multimers for the sensitive, direct detection of human hepatitis viruses. *Nucleic Acids Symp Ser*, 24: 197–200.

336. Vallverdú-Queralt A, Regueiro J, Alvarenga, JFR, Martinez-Huelamo M, Leal LN, Lamuela-Raventos RM (2015). Characterization of the phenolic and antioxidant profiles of selected culinary herbs and spices: caraway, turmeric, dill, marjoram and nutmeg. *Food Sci Technol*, 35(1): 189–195.

337. van den Bout-van den Beukel CJ, Hamza OJ, Moshi MJ, Matee MI, Mikx F, Burger DM, Koopmans PP, Verweij PE, Schoonen WG, van der Ven AJ (2008). Evaluation of cytotoxic, genotoxic and CYP450 enzymatic competition effects of Tanzanian plant extracts traditionally used for treatment of fungal infections. *Basic Clin Pharmacol Toxicol*, 102(6): 515–26.

338. van Rossum TG, Vulto AG, de Man RA, Brouwer JT, Schalm SW (1998). Review article: glycyrrhizin as a potential treatment for chronic hepatitis C. *Aliment Pharmacol Ther*, 12(3): 199–205.

339. van Wyk BE, Albrecht C (2008). A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). *J Ethnopharmacol*, 119: 620–629.

340. Varghese A, Pandita N, Gaud RS (2014). *In vitro* and *in vivo* evaluation of CYP1A interaction potential of *Terminalia Arjuna* Bark. *Indian J Pharm Sci*, 76(2): 138–147.

341. Vavricka SR, Van Montfoort J, Ha HR, Meier PJ, Fattinger K (2002). Interactions of rifamycin SV and rifampicin with organic anion uptake systems of human liver. *Hepatology*, 36(1): 164–72.

342. ven Murthy MR, Ranjekar PK, Ramassamy C, Deshpande M (2010). Scientific basis for the use of Indian ayurvedic medicinal plants in the treatment of neurodegenerative disorders: *ashwagandha*. *Cent Nerv Syst Agents Med Chem*, 10(3): 238–46.

343. Venkatesan K (1989). Clinical pharmacokinetic considerations in the treatment of patients with leprosy. *Clin Pharmacokinet*, 16(6): 365–86.

344. Walsky RL, Obach RS (2004). Validated assays for human cytochrome P450 activities. *Drug Metab Dispos*, 32(6): 647–60.

345. Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M, LeCluyse EL (2003). A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem*, 278(16): 14146–52.

346. Wang S, Liu L, Wang L, Hu Y, Zhang W, Liu R (2012). Structural characterization and identification of major constituents in *Jitai* tablets by high-performance liquid chromatography/diode-array detection coupled with electrospray ionization tandem mass spectrometry. *Molecules*, 17: 10470–10493.

347. Wang Y, Wu S, Chen Z, Zhang H, Zhao W (2015). Inhibitory effects of cytochrome P450 enzymes CYP1A2, CYP2A6, CYP2E1 and CYP3A4 by extracts and alkaloids of *Gelsemium elegans* roots. *J Ethnopharmacol*, 166: 66–73.

348. Wanwimolruk S, Phopin K, Prachayasittikul V (2014). Cytochrome P450 enzyme mediated herbal drug interactions (Part 2). *EXCLI J*, 13: 869–896.

349. Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z (2003). The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J Pharmacol Exp Ther*, 306(1): 287–300.

350. Waxman DJ, Azaroff L (1992). Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J*, 281(3): 577–92.

351. Wei BH, Ye J, Xue BJ, Sun GX, Yang XY, Zhang YJ (2014). Effect of continuous oral cycloastragenol on the activity of cytochrome P450 enzymes in rats. *Chinese J New Drugs*, 23: 476–479.

352. Wen X, Walle UK, Walle T (2005). 5,7-Dimethoxyflavone downregulates CYP1A1 expression and benzo[a]pyrene-induced DNA binding in Hep G2 cells. *Carcinogenesis*, 26(4): 803–9.

353. Wen X, Wang JS, Neuvonen PJ, Backman JT (2002). Isoniazid is a mechanism-based inhibitor of cytochrome P450 1A2, 2A6, 2C19 and 3A4 isoforms in human liver microsomes. *Eur J Clin Pharmacol*, 57(11): 799–804.

354. White RE (2012). Review of drug metabolism in drug discovery and development. Encyclopaedia of drug metabolism and interactions. I: 1–40.

355. Wilkinson GR (2005). Drug metabolism and variability among patients in drug response. *N Engl J Med*, 352(21): 2211–2221.

356. Williams J, Bauman JN, Cai H, Conlon K, Hansel S, Hurst S, Sadagopan N, Tugnait M, Zhang L, Sahi J (2005). *In vitro* ADME phenotyping in drug discovery: Current challenges and future solutions. *Curr Opin Drug Discov Devel*, 8: 78–88.

357. Williams KJ, Piddock LJ (1998). Accumulation of rifampicin by *Escherichia coli* and *Staphylococcus aureus*. *J Antimicrob Chemother*, 42(5): 597–603.

358. Williamson JS, Wyandt CM (1997). Herbal therapies: The facts and the fiction. Drug topics, Medical Economics, USA, 141: 78–85.

359. Wilson D, Goggin K, Williams K, Gerkovich MM, Gqaleni N, Syce J, Bartman P, Johnson Q, Folk WR (2015). Consumption of *Sutherlandia frutescens* by HIV-seropositive South African adults: An adaptive double-blind randomized placebo controlled trial. *PLoS One*, 10(7): e0128522.

360. Wintola A, Afolayan AJ (2017). Chemical constituents and biological activities of essential Oils of *Hydnora africana* thumb used to treat associated infections and diseases in South Africa. *Appl Sci*, 7: 443.

361. WHO TB guidelines (2017). World Health Organization - Guidelines for the treatment of drug-susceptible tuberculosis and patient care, 2017 update, WHO/HTM/TB/2017.05, ISBN 978-92-4-155000-0.

362. WHO TMS (2014). WHO traditional medicine strategy. Available at: *http://apps.who.int/iris/bitstream/10665/92455/1/9789241506090_eng.pdf?ua=1*.

363. Wyk VP, Msemburi W, Laubscher R, Dorrington RE, Groenewald P, Glass T, Nojilana B, Joubert JD, Matzopoulos R, Prinsloo M, Nannan N, Gwebushe N, Vos T, Somdyala N, Sithole N, Neethling I, Nicol E, Rossouw A, Bradshaw D (2013). Second national burden of disease study South Africa: national and subnational mortality trends, 1997–2009. *The Lancet*, 381: s113.

364. Xinhuanet (2016). Traditional Chinese medicine in China. Available from: *http://news.xinhuanet.com/english/china/2016-12/06/c_135883982.htm*.

365. Xu C, Desta Z (2013). *In vitro* analysis and quantitative prediction of efavirenz inhibition of eight cytochrome P450 (CYP) enzymes: Major effects on CYPs 2B6, 2C8, 2C9 and 2C19. *Drug Metab Pharmacokinet*, 28(4): 362–371.

366. Xu C, Ogburn ET, Guo Y, Desta Z (2012). Effects of the CYP2B6*6 allele on catalytic properties and inhibition of CYP2B6 *in vitro*: Implication for the mechanism of reduced efavirenz metabolism and other CYP2B6 substrates *in vivo*. *Drug Metab Dispos*, 40(4): 717–725.

367. Yamashita F, Sasa Y, Yoshida S, Hisaka A, Asai Y, Kitano H, Hashida M, Suzuki H (2013). Modeling of rifampicin-induced CYP3A4 activation dynamics for the prediction of clinical drug-drug interactions from *in vitro* data. *PLoS ONE*, 8(9): e70330.

368. Yanakakis LJ, Bumpus NN (2012). Biotransformation of the antiretroviral drug etravirine: metabolite identification, reaction phenotyping, and characterization of autoinduction of cytochrome P450-dependent metabolism. *Drug Metab Dispos*, 40(4): 803–14.

369. Yang NY, Duan JA, Shang EX, Tian LJ (2010). Analysis of sesquiterpene lactones in *Eupatorium lindleyanum* by HPLC-PDA-ESI-MS/MS. *Phytochem Anal*, 21(2): 144–149.

370. Yang Z, Garcia A, Songli Xu, Powell RD, Vertino PM, Singh S, Marcus AI (2013). *Withania somnifera* root extract inhibits mammary cancer metastasis and epithelial to mesenchymal transition. *PLOS ONE*, 8(9): e75069.

371. Yanitskaya AV, Mitrofanova IY, Shulenina YS (2012). Phytochemical basis of raw material in *Inula helenium* herb growing in Volgograd region. *Volgograd J Med Res*, 1(33): 24–26.

372. Yasui S, Fujiwara K, Tawada A, Fukuda Y, Nakano M, Yokosuka O (2011). Efficacy of intravenous glycyrrhizin in the early stage of acute onset autoimmune hepatitis. *Dig Dis Sci*, 56(12): 3638–47.

373. Ye M, Guo D, Ye G, Huang C (2005). Analysis of homoisoflavonoids in *Ophiopogon japonicus* by HPLC-DAD-ESI-MSn1. *J Am Soc Mass Spectrom*, 16(2): 234–243.

374. Yu AM, Qu J, Felmlee MA, Cao J, Jiang XL (2009). Quantitation of human cytochrome P450 2D6 protein with immunoblot and mass spectrometry analysis. *Drug Metab Dispos*, 37: 170–177.

375. Yu K, Chen F, Li C (2012). Absorption, disposition, and pharmacokinetics of saponins from Chinese medicinal herbs: what do we know and what do we need to know more? *Curr Drug Metab*, 13(5): 577–98.

376. Yu T, Chen X, Wang Y, Zhao R, Mao S (2014). Modulatory effects of extracts of vinegar-baked *Radix Bupleuri* and saikosaponins on the activity of cytochrome P450 enzymes *in vitro*. *Xenobiotica*, 44(10): 861–867.

377. Yao M, Zhu M, Sinz MW, Zhang H, Humphreys WG, Rodrigues AD, Dai R (2007). Development and full validation of six inhibition assays for fi ve major cytochrome P450 enzymes in human liver microsomes using an automated 96 - well microplate incubation format and LC - MS/MS analysis. *J Pharm Biomed Anal*, 44: 211–223.

378. Zanger UM, Klein K, Saussele T, Blievernicht J, Hofmann MH, Schwab M (2007). Polymorphic CYP2B6: molecular mechanisms and emerging clinical significance. *Pharmacogenomics*, 8(7): 743–59.

379. Zanger UM and Kathrin Klein (2013). Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance. *Front Genet*, 4: 24.

380. Zanger UM, Klein K, Saussele T, Blievernicht J, Hofmann MH, Schwab M (2007). Polymorphic CYP2B6: molecular mechanisms and emerging clinical significance. *Pharmacogenomics*, 8(7): 743–59.

381. Zhang D, Chando TJ, Everett DW, Patten CJ, Dehal SS, Humphreys WG (2005). *In vitro* inhibition of UDP glucuronosyltransferases by atazanavir and other HIV protease inhibitors and the relationship of this property to *in vivo* bilirubin glucuronidation. *Drug Metab Dispos*, 33(11): 1729–39.

382. Zhang D, Surapaneni S (2012). ADME-Enabling Technologies in Drug Design and Development, John Wiley & Sons, Inc., USA, pp. 1–622.

383. Zhang JW, Liu Y, Cheng J, Li W, Ma H, Liu HT, Sun J, Wang LM, He YQ, Wang Y, Wang ZT, Yang L (2007). Inhibition of human liver cytochrome P450 by star fruit juice. *J Pharm Pharmaceut Sci*, 10(4): 496–503.

384. Zhang M, Liu WX, Zheng MF, Xu QL, Wan FH, Wang J, Lei T, Zhou ZY, Tan JW (2013). Bioactive quinic acid derivatives from *Ageratina adenophora*. *Molecules*, 18: 14096–14104.

385. Zhang Y, Huang L, Bi H, Cui Y, Li J, Wang X, Qin X, Chen J, Huang M (2013). Study of the upregulation of the activity of cytochrome P450 3A isoforms by Astragalus injection and Astragalus granules in rats and in cells. *Eur J Drug Metab Pharmacokinet*, 38(2): 105–113.

386. Zhang Y, Zhang A, Zhang Y, Sun H, Meng X, Yan G, Wang X (2016). Application of ultra-performance liquid chromatography with time-of-flight mass spectrometry for the rapid analysis of constituents and metabolites from the extracts of *Acanthopanax senticosus* Harms Leaf. *Pharmacogn Mag*, 12(46): 145–52.

387. Zhao K, Ding M, Cao H, Cao ZX (2012). In-vitro metabolism of glycyrrhetinic acid by human and rat liver microsomes and its interactions with six CYP substrates. *J Pharm Pharmacol*, 64(10): 1445–51.

388. Zhao P (2012). Update of 2012 FDA draft guidance on drug interactions: model-based strategy to integrate knowledge and quantify effects, Center for Drug Evaluation and Research Food and Drug Administration, Land O' Lakes conference on drug metabolism/applied pharmacokinetics, Merrimac, WI, USA.

389. Zhao Y, Zhang M, Shi Q, Kiyota H (2006). Chemical constituents of plants from the genus *Inula*. *Chem Biodivers*, 3: 371–384.

390. Zhou H, Meibohm B (2013). Drug-drug interactions for therapeutic biologics, Wiley publications, John Wiley & Sons, Inc., USA, ISBN: 978-1-118-03216-9, 386p.

391. Zhu Z, Kim S, Chen T, Lin JH, Bell A, Bryson J, Dubaquie Y, Yan N, Yanchunas J, Xie D, Stoffel R, Sinz M, Dickinson K (2004). Correlation of high - throughput pregnane X receptor (PXR) transactivation and binding assays. *J Biomol Screen*, 9: 533–540.

392. Zöllner T, Schwarz M (2013). Herbal Reference Standards: applications, definitions and regulatory requirements. *Rev Bras Farmacog*, 23(1): 1–21.

Appendix 1

Abstracts of paper published from this thesis

Appendix 1.1: Simultaneous HPLC Determination of Efavirenz, 8-Hydroxy Efavirenz, Neostigmine and Comparison of their Separation Using a C18 and Biphenyl Column through Pharmacological Evaluation **DOI: 10.4172/pharmaceutical-sciences.1000237**

Research Paper

Simultaneous HPLC Determination of Efavirenz, 8-Hydroxy Efavirenz, Neostigmine and Comparison of Separation Using a C18 and Biphenyl Column through Pharmacological Evaluation

S. KUMAR*, P. J. BOUIC[1,2] AND B. ROSENKRANZ

Division of Clinical Pharmacology, Department of Medicine, University of Stellenbosch, Cape Town, 7505, [1]Synexa Life Sciences, Montague Gardens, Cape Town, 7441, [2]Division of Medical Microbiology, Department of Medicine, University of Stellenbosch, Cape Town, 7505, South Africa

A simple, rapid and stable high performance liquid chromatography method for a combination of efavirenz, its major metabolite 8-hydroxy efavirenz and neostigmine was developed and validated. The drugs individually and in combination, were analysed using an Agilent 1260 high performance liquid chromatography coupled with variable wavelength detector. Successful separation of combined drugs were achieved by gradient elution on a reverse-phase C18 Phenomenex Evo 100A column (150×4.6 mm, 2.6 µ), by gradient elution using a mobile phase consisting of water:acetonitrile at 0.6 ml×min^{-1} flow rate, detection wavelength 245 nm, column oven temperature 27° and injection volume 15 µl. The same method was also deployed on a Restek Ultra biphenyl column (150×4.6 mm, 5 µ) to compare the levels of separation of the drugs and the metabolite. The chromatographic retention times were consistent at 8.75, 9.33 and 10.20 min for efavirenz, 8-hydroxy efavirenz and neostigmine, respectively. Polynomial regression data for the calibration plots exhibited linear relationship (correlation coefficient=0.999) in the range of 2.5-150 µM for both efavirenz and 8-hydroxy efavirenz, and the lower limit of detection and lower limit of quantification at 32 and 96.97 µM for efavirenz and 29.49 and 89.38 µM for 8-hydroxy efavirenz, respectively. Evaluation of drug metabolism using human liver microsomes could be achieved with this method and linearity was established for the 30, 45 and 60 min incubations (correlation coefficient=0.97). The method could be a potential tool to investigate herb and drug-drug interactions as well as for quantifying drugs in *in vitro* drug metabolism assays.

Key words: HPLC, C18, efavirenz, 8-hydroxy efavirenz, neostigmine, biphenyl column, human liver microsomes

Efavirenz (EFV, $C_{14}H_9ClF_3NO_2$) is an antiretroviral (ARV) agent employed in the treatment of human immunodeficiency virus (HIV) type 1 in combination with other drugs. It is an analogue of non-nucleoside ARV agent and a non-competitive reverse transcriptase inhibitor and is directly connected to the enzyme and blocks RNA and DNA-dependent, DNA-polymerase activities, causing destruction of the enzyme catalytic site. Chemically it is (S)-6-chloro-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one and is optically active with a molecular weight of 315.68 g/mol and melting point in the range 139-141°. This crystalline powder has a white or slightly yellowish appearance. The substance has a melting point ranging from 136-141°, is nearly water insoluble but soluble in methanol and dichloromethane[1]. EFV is predominantly cleared by hepatic metabolism[2]. The metabolites identified in human plasma and urine (almost exclusively as glucuronide or sulphate conjugates) were 7- and 8-hydroxy efavirenz (primary metabolites) and 8,14-dihydroxy efavirenz (secondary metabolite). 8-hydroxy efavirenz (E8H, $C1_4H_9ClF_3NO_3$) is the major metabolite from EFV primarily formed via CYP2B6 of the cytochrome P_{450} system[3]. It has the IUPAC name (4S)-6-chloro-4-(2-

*Address for correspondence
E-mail: saneesh.7.kumar@gmail.com

Accepted 06 April 2017
Revised 31 December 2016
Received 13 September 2016
Indian J Pharm Sci 2017;79(3):353-360

Appendix 1.2: A validated stable HPLC method for the simultaneous determination of rifampicin and 25-O-desacetyl rifampicin – evaluation of *in vitro* metabolism

DOI: 10.1556/1326.2018.00361

Original Research Paper

A Validated Stable HPLC Method for the Simultaneous Determination of Rifampicin and 25-*O*-Desacetyl Rifampicin – Evaluation of *in vitro* Metabolism

Saneesh Kumar[1*], Patrick J. Bouic[2,3] and Bernd Rosenkranz[1]

[1]*Division of Clinical Pharmacology, Department of Medicine, University of Stellenbosch, Tygerberg, Cape Town, South Africa*
[2]*Division of Medical Microbiology, Faculty of Health Sciences, University of Stellenbosch, Cape Town, RSA*
[3]*Synexa Life Sciences, Montague Gardens, Cape Town, RSA*

Received: 27 July 2017; accepted: 10 December 2017

A simple, efficient, and stable high-performance liquid chromatography (HPLC) separation method for a combination of rifampicin (RIF), its major metabolite 25-*O*-desacetyl rifampicin (25ODESRIF), and neostigmine (NEO) was developed and validated. The drugs individually, and in combination, were analyzed using a Waters Alliance 2695 HPLC coupled with 2996 photodiode array detector (PDA). Successful separation of combined drugs was achieved by gradient elution on a reverse-phase C-18 Phenomenex Luna column, using a mobile phase consisting of water and methanol at detection wavelength of 254 nm. The HPLC retention times were consistent at ±7.70 min, ±8.25 min, and ±10.70 min for RIF, 25ODESRIF, and NEO, respectively. The regression data for the calibration plots exhibited linear relationship (R^2 = 0.995) in the range of 0–200 μM for both RIF and 25ODESRIF, and the lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were calculated at 5.86 μM and 17.75 μM for RIF and 7.78 μM and 23.57 μM for 25ODESRIF, respectively. The method was evaluated using in vitro human liver microsomes (HLMs) assays, and linearity was established for the 15, 30, 45, and 60 min incubations (R^2 = 0.99). The formation of 25ODESRIF was characterized by hyperbolic kinetics (K_m 48.23 μM, V_{max} 1.233 pmol/min/mg protein, and CL_{int} 0.026 μl/min/mg protein). The method was applied in HLM assays to understand the herb–drug interaction (HDI) potential of *Althaea officinalis*, a popular African herb consumed by tuberculosis (TB) patients, with RIF. None of the extracts of *A. officinalis* inhibited the esterase-mediated metabolism pathway of RIF, compared to the positive control nelfinavir (IC_{50} = 9.59 μM). The method provides a tool for quantifying RIF and 25ODESRIF in in vitro drug metabolism assays as well as investigating herb– and drug–drug interactions (DDIs).

Keywords: HPLC, C-18, rifampicin, 25-*O*-desacetyl rifampicin, neostigmine, human liver microsomes, Michaelis–Menten kinetics, *Althaea officinalis*, herb–drug interaction

Introduction

Rifampicin (RIF) (3-[4-methyl piperazinyl-iminomethyl] rifamycin SV; rifampin) is a first-line antibiotic along with isoniazid (INH), pyrazinamide (PYR), ethambutol (ETB), and streptomycin (STP) in the treatment of pulmonary and extrapulmonary tuberculosis (TB) and has a unique role in the killing of semi-dormant tubercle bacilli (*Mycobacterium tuberculosis*) [1].

The World Health Organization recommends a 6-month regimen comprising RIF, INH, PYR, and ETB which are given together for the first 2 months followed by RIF and INH therapy for the next 4 months. RIF is mainly eliminated in the bile and then reabsorbed; hence, enterohepatic circulation ensues. The drug is deacylated into its microbiologically active metabolite 25-*O*-desacetyl rifampicin (25ODESRIF), by esterases or by enzymes present in microsomal cells [2]. 25ODESRIF is less absorbable as compared to the parent drug [3].

RIF is used as an inducer of cytochrome P450 3A4 (CYP 3A4) to analyze the effect of herbal medicine on the activity of this enzyme [4, 5]. It induces gut and liver (hepatic) CYP3A4 and autoinduces its own metabolism [6]. P-glycoprotein (P-gp), along with B-esterases, is involved in disposition of rifampicin [7].

Very few high-performance liquid chromatography (HPLC) methods have been developed for the quantification of RIF and its metabolite 25ODESRIF, in plasma [8–10] and urine [11–14]. These HPLC methods involve various procedures which can be time-consuming. This is of critical importance especially in assays involving HLMs and other liver fractions (S9, hepatocytes), to identify the metabolites and also quantify the rate of drug metabolism, as well as for major pharmacokinetic studies as well as herb–drug interaction (HDI) and drug–drug interaction (DDI) studies. Metabolites may interfere with the assay when analyzing parent drugs [15]. Rifampicin induces the metabolism of many drugs, including antiretroviral drugs (ARVs) such as nevirapine metabolized by the CYPs, predominantly CYP3A4 and CYP2B6 [16, 17]. Few in vitro studies have assessed K_m and V_{max} kinetic parameters of rifampicin in human liver microsomes.

RIF-induced CYP3A4 activation and expression models have been used for predicting the pharmacokinetics of CYP3A4 substrate drugs as well as clinically relevant DDI [18]. Most of the in vitro HDI studies have investigated the role of RIF as an inducer of CYP3A4 along with other drugs and herbal extracts; less information is available on the effect of herbal medicines on the esterase-mediated metabolism pathway of RIF to 25ODESRIF. Assessment of the metabolic disposition of RIF and of possible inhibitory/inducing effects of herbal medicines would be very important.

Althaea officinalis L. (Malvaceae), commonly called "marshmallow" (Baer-ul-Khtmk [African/Arabic]; Gulkhairo [Pers.]), is a perennial species indigenous to Africa, which is used as an ornamental plant and a medicinal plant by TB patients [19–21].

* Author for correspondence: saneesh.7.kumar@gmail.com

DOI: 10.1556/1326.2018.00361

Appendix 1.3: Abstract Book: Innopharm 2. Proceedings: 2nd International Conference on Bridging Innovations in Pharma. Med. & Bio Sciences. International Journal of Pharmacy and Pharmaceutical Sciences, [S.l.], p. 1-255, Feb. 2017. ISSN 0975-1491.

HPLC/lC-MS GUIDED PHYTOCHEMICAL/*IN VITRO* SCREENING OF INULA HELENIUM L. (*ASTERACEAE*) AND ALTHAEA OFFICINALIS L. (*MALVACEAE*), AND PREDICTION OF POSSIBLE CYTOCHROME P450 INTERACTIONS

SANEESH KUMAR*[1], NONTOMBI SEPHULE[3], CHARLES AWORTWE[1], PATRICK J. BOUIC [2, 3], BERND ROSENKRANZ[1]

[1]Division of Clinical Pharmacology, Department of Medicine, University of Stellenbosch, Cape Town, RSA, [2]Division of Medical Microbiology, Faculty of Health Sciences, University of Stellenbosch, Cape Town, RSA, [3]Synexa Life Sciences, Montague Gardens, Cape Town, RSA
Email: saneesh.7.kumar@gmail.com

Abstract

The dried roots of *Inula helenium* L. (Asteraceae) and *Althaea officinalis* L. (Malvaceae) are used as traditional medicines for various medical conditions including tuberculosis, in Africa. The aim of this study is to determine the major phytoconstituents present in the roots of *I. helenium* and *A. officinalis* and assess their potential in altering the activity of cytochrome P450 enzymes, through *in vitro* assays using Human liver microsomes (HLM). Aqueous, methanolic, and ethanolic extracts of *I. helenium* and *A. officinalis* were analysed using biochemical tests, HPLC-VWD and LC-ESCI-MS using quercetin (flavonoids), caffeine (alkaloids), 1-benzopyran-2-one (coumarins), lanatoside C (glycosides), and gallic acid (phenols) as analytical reference standards. *In vitro* inhibition assays were done using HLM with Rifampicin as the substrate for the target enzyme CYP3A4. The biochemical tests confirmed the presence of alkaloids, saponins, phenols, glycosides, terpenoids, flavonoids and coumarins in almost all plants, especially the methanolic extracts. The HPLC retention times were consistent with the presence of quercetin, coumarin, lanatoside C, caffeine and gallic acid standards (retention times 0.71 min, 1.23 min, 1.93 min, 1.25 min and 0.79 min respectively; plant extracts with mean retention times±0.67 min,±1.09 min,±0.61 min,±0.39 min and±0.79 min). LC-ESCI-MS analysis further confirmed this through the MRM scans. Glycosides were observed in almost all plants. In *I. helenium*, the prolific compound observed was alantolactone (*helenin*) or isoalantolactone which belonged to the class of sesquiterpene lactones. In *A. officinalis*, the known lactone, n-hexacos-2-enyl-1,5-olide (althea hexacosanol lactone) was observed at 387.39 *m/z* along with quercetin-equivalent flavonoids which could be potential inhibitors of CYP3A4 and 2B6. Positive/negative mode full scans also showed the presence of major compounds such as sesquiterpene lactones, flavones, saponins and amides. HPLC-guided HLM screening assays indicated the inhibitory potential of the aqueous and methanolic extracts of *I. helenium* on CYP3A4 (IC_{50} = 108 µg/ml and 42.79µg/ml, respectively) compared to the methanolic extract of *A. officinalis* (IC_{50}> 150 µg/ml). The result suggests that co-administration of the various extracts of *I. helenium* and *A. officinalis* with anti-TB drugs that are substrates of CYP3A4, 2B6, 2C9 and 2C19 enzymes could, in turn, lead to undesirable pharmacokinetic herb-drug interactions in humans.

Keywords: HPLC/lC-ESCI-MS fingerprinting, elecampane, marshmallow, cytochrome P450, phytoconstituents, human liver microsomes

Appendix 1.4: Abstract Book: 1st International Conference on Novel Frontiers in Pharmaceutical & Health Sciences (Innopharm1). International Journal of Pharmacy and Pharmaceutical Sciences, [S.I.], Sep. 2015. ISSN 0975-1491.

HPLC/lC-MS GUIDED PHYTOCHEMICAL SCREENING OF *ASTRAGALUS MEMBRANACEUS*, AND PREDICTION OF POSSIBLE CYTOCHROME P450 INTERACTIONS

Saneesh Kumar[a*], Nontombi Sephule[c], Charles Awortwe[a], Patrick J. Bouic[b,c], Bernd Rosenkranz[a]

[a]Division of Clinical Pharmacology, Department of Medicine, University of Stellenbosch, Cape Town, RSA, [b]Division of Medical Microbiology, Faculty of Health Sciences, University of Stellenbosch, Cape Town, RSA, [c]Synexa Life Sciences, Montague Gardens, Cape Town, RSA
***Email: saneesh.7.kumar@gmail.com**

Abstract

The dried roots of *Astragalus membranaceus* (Fabaceae) are used as folklore medicine for multifarious diseases including HIV/AIDS, in Africa. Objective of study was to determine the phytoconstituents present in the roots of A. *membranaceus*, using methanol, ethanol, aqueous and ethyl acetate solvent extractions, and assess the potential of each extract in altering the activity of cytochrome P450 enzymes. Exhaustive extraction, of the dried roots of A. *membranaceus*, was done using water, methanol, ethanol and ethyl acetate, and qualitative analysis was completed using biochemical tests, HPLC analysis and multiple reaction monitoring HPLC combined with electrospray ionisation and tandem MS (HPLC-ESCI/MS/MS) using quercetin, caffeine, coumarin, lanatoside C, and gallic acid as reference standards for flavonoids, alkaloids, coumarins, cardiac glycosides, and phenols. The biochemical tests confirmed the presence of alkaloids, saponins, phenols, glycosides, terpenoids, flavonoids and coumarins in almost all extracts, the methanol extracts having the most, compared to the other solvent extracts. The HPLC analysis baselined quercetin, coumarin, lanatoside C, caffeine, and gallic acid standards with retention times 0.71 min, 1.23 min, 1.93 min, 1.25 min and 0.79 min respectively, and all the extracts with retention times±0.62 min,±1.0 min,±0.56 min,±0.36 min and±0.78 min on the average, with respect to their equivalent standards. The LC/MS analysis further confirmed the presence of flavonoids, phenols, glycosides and coumarins in most extracts with MRM scan retention times in close proximity with the masses of the daughter ions of the standards; 1.64 min for quercetin, 2.44 min for caffeine, 6.92 min for lanatoside C, 2.08 min for coumarin and 1.3 min for gallic acid. The methanol extract had lanatoside C equivalent with MRM daughter ion scan retention time matching at 6.94 min. The ethyl acetate extract had alkaloids that matched caffeine MRM daughter mass scan range, though at a different retention time. Full scans both positive and negative ion modes, also showed the presence of many phytoconstituents in the same class of compounds as the internal standards. The assessment model baselined flavonoids (quercetin) as potential inducers, terpenoids and coumarins as potential inhibitors, the alkaloid caffeine as an inducer and glycoside derivates as inhibitor. The phytochemical fingerprints of all the extracts from A. *membranaceus* projected the possibility of its inhibitory/inducive effect on the cytochrome P450 enzymes. In conclusion, the results show that the consumption of A. *Membranaceus* together with conventional drugs may present a risk of possibly relevant herb-drug interaction.

Keywords: *Astragalus*, HPLC/lC-MS, phytoconstituents, fingerprint, cytochrome P450, flavonoids, glycosides, coumarin.

Appendix 2

Ethics exemption approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY

jou kennisvennoot • your knowledge partner

Ethics Letter

19-May-2015

Ethics Reference #: X15/04/002
Clinical Trial Reference #:
Title: Pharmacokinetic herb-drug interactions involving African traditional medicines - fingerprint analysis and in vitro metabolism studies.

Dear Mr Saneesh Kumar,

Thank you for your application to our Health Research Ethics Committee (HREC). This application is for laboratory research which does not involve any animal or human subjects.

The Health Research Ethics Committee considers this proposal to be exempt from ethical review.

This letter confirms that this research is now registered and you can proceed with study related activities.

If you have any queries or need further assistance, please contact the HREC Office 0219399657.

Sincerely,

REC Coordinator
Franklin Weber
Health Research Ethics Committee 1